Enhanced biocatalytic sustainability of laccase by immobilization on functionalized carbon nanotubes/polysulfone membranes

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Abstract

Chemically functionalized multi-walled carbon nanotubes (CNTs) are used as carriers for laccase immobilization. In this work, CNTs were modified using different approaches with a combination of methods involving hydrothermal oxidation with nitric acid, treatment with 3-aminopropyltriethoxysilane, glutaraldehyde, N-ethyl-N-(3-(dimethylamino)-propyl) carbodiimide hydrochloride and N-hydroxysuccinimide. The enzyme immobilization efficiency and recovered activity were evaluated towards 2,2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic acid) biocatalytic oxidation. The best compromise between immobilization efficiency and recovered activity was obtained using the CNTs functionalized with 0.3 M HNO_3, treated with N-ethyl-N-(3-(dimethylamino)propyl) carbodiimide hydrochloride and N-hydroxysuccinimide. This catalyst also showed the best thermal stability (at 50 and 60 ºC). The bioconjugate based on this material was characterized by vibrational spectroscopies (FTIR and Raman) and by N_2 adsorption. The results from reutilization tests showed that laccase activity was kept above 65% of its initial value after five consecutive cycles of reuse. The biocatalytic performance of the immobilized enzyme was evaluated for the degradation of a mixture of phenolic compounds in water containing phenol, resorcinol, 4-methoxyphenol and 4-chlorophenol. As means of cost efficient to enzyme reutilization, laccase was immobilized over polysulfone membranes blended with the functionalized CNTs and studied in the degradation of 4-methoxyphenol.

Keywords*: Multi-walled carbon nanotubes (CNTs); Chemical functionalization; Laccase; immobilization; CNTs-based polysulfone membranes; Wastewater treatment.*

1. Introduction

Enzyme immobilization was primarily introduced to avoid the waste of costly enzymes, allowing their reuse in many processes. Following the early attempts to produce stable systems, the applications of immobilized enzymes are continuously increasing [1]. One of the smartest uses of immobilized enzymes is their application in the treatment of wastes, including waste waters [2-6] where both the specific activity and possibility of recycle are combined for maximum performance [7].

Interestingly, carbon materials have been long preferred as carriers for enzyme immobilization, mainly because they may provide a large surface area relative to the enzyme loading. In what concerns carbon nanotubes (CNTs), their exclusive structure and properties have been intensively studied in distinct applications ranging from energy storage, biotechnology and environmental remediation [8]. Additionally, CNT surface can be easily functionalized, tuning their properties towards specific applications and enhancing their efficiency either as supports or catalysts.

The use of CNTs as supports for enzyme immobilization is reported for several applications such as bio-sensing, water remediation and biodiesel production [9-13]. CNTs present exceptional advantages when compared to traditional supports (such as silica, polymeric materials, etc.), including the large specific surface area, high adsorption capacity, and a superior enzyme loading capacity (even as compared to other carbon materials). In general, free enzymes can be destabilized or even deactivated when submitted to severe conditions of pH and temperature and/or in contact with organic solvents [14]. The immobilization of enzymes on solid carriers, and particularly on CNTs, is considered an effective strategy for improving the long-term operational performance, stability, shelf-storage life, selectivity, and last but not least, reusability [12, 13]. Several approaches for enzyme immobilization on CNTs, including

adsorption, covalent bonding, entrapment or encapsulation, have been described in the literature [15]. However, the possibility of enzyme leaching during the catalytic cycles has limited the use of immobilization techniques at laboratorial and industrial scale. Moreover, it is expected that the CNT-enzyme hybrids may present lower activity than the free form of the enzyme. Thus, the investigation on the interactions between the enzyme and the support is fundamental for the development of highly active and stable biocatalysts. Furthermore, the immobilization of enzymes in membranes may provide an increased availability of enzyme to the substrate, low leaching, prolonged lifetime, enhanced mechanical strength and reusability, among others. Functionalized CNT-based membranes have been explored mostly for the development of biosensors and for the biocatalytic treatment of contaminated water [12, 13, 16], in particular CNT-enzyme membranes avoids the costly and time-consuming post-separation of the biocatalyst and also allows the operation in continuous flow or multi-batch regime [5, 13, 17-19]. Polysulfone (PSf) membranes are widely used in water applications due to their excellent heat resistance and chemical stability over a large range of pH [20].

Laccase (EC 1.10.3.2) is a multi-copper blue oxidase enzyme found in higher plants and fungi, as well as in insects and bacteria [21, 22]. This enzyme is responsible for the oxidation of a broad range of organic and some inorganic substrates [23]. Laccase and the laccase-mediator systems have great biotechnological potential resulting from both the large variety of possible reactions and the broad substrate specificity. Potential applications include textile dye and pulp bleaching, food enhancement, bioremediation of soils and water, polymer synthesis, and development of biosensors and biofuel cells [24-27]. The principal bottlenecks of laccase-based biocatalytic processes are the low stability of the enzyme associated to its high cost [28]. The immobilization of laccase can be considered the best of two worlds, since the

separation and reuse of this costly biocatalyst becomes possible, while maintaining, or even enhancing its catalytic activity. As mentioned above, there are already studies reporting the use of enzymes immobilized both on PSf and on CNT-based membranes [29-32]. Yet, to the best of our knowledge, the use of CNT-PSf membranes for laccase immobilization has not been reported in the literature. In addition, in this work, CNT surface modification was proposed as a successful strategy to improve enzyme reuse for the removal of toxic compounds, and at the same time. For this purpose, multi-walled carbon nanotubes (CNTs) were functionalized using different techniques to promote the immobilization of laccase. The immobilization efficiency, biocatalytic activity and reusability of the resulting bioconjugates were assessed towards the oxidation of the typical laccase substrate 2,2'-azino-*bis*(3-ethylbenzathiazoline-6-sulfonic acid), ABTS. Finally, for the first time, the degradation of phenolic compounds in water was carried out using the best performing CNT-laccase bioconjugate both in powder form and incorporated on PSf membranes.

2. Experimental

2.1 Materials, enzyme and chemicals

Multi-walled CNTs were obtained from Shenzhen Nanotechnologies Co. Ltd. (purity ≥ 95%; 10–20 nm diameter; 5–15 μm length; ash content ≤ 0.2 wt%, amorphous carbon < 3%: surface area = 73 m^2 g^{-1}). Commercial laccase (Novozym® 51003; 1000 LAMU/g), obtained from genetically modified *Aspergillus oryzae*, was kindly donated by Novozymes (Denmark). 2,2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic acid) (ABTS, 98%), (3-aminopropyl)triethoxysilane (APTES, 99%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, ≥ 98%), nitric acid (HNO_3, ≥ 65%), tetrahydrofuran (THF, ≥ 99.9%), phenol (PH, ≥ 99%), resorcinol (RS, 99%)

polyvinylpyrrolidone (PVP, 10 kDa), 1 methyl-2-pyrrolidinone (NMP, 99.5%) and 4-chlorophenol (CP, ≥ 99%) were purchased from Sigma–Aldrich. Glutaraldehyde (30%) was obtained from Merck. Thionyl chloride ($SOCl_2$, ≥ 99.0%) and 4-methoxyphenol (MP, ≥ 98%) were purchased from Fluka. N-hydroxysuccinimide (NHS, 98%) was obtained from Alfa Aesar. Polysulfone Udel® P-3500 LCD MB3 polymer was kindly supplied in pellet form by Solvay.

2.2 CNTs chemical functionalization

2.2.1 Liquid-phase oxidation

Hydrothermal oxidation of the pristine CNTs was performed in a Teflon-lined stainless steel autoclave using HNO_3 aqueous solutions with variable concentrations (0.05, 0.10, 0.20 and 0.30 M) at 200 ºC, as described elsewhere [33]. Briefly, 0.2 g of CNTs was added to 75 mL of a HNO_3 aqueous solution. After being sealed, the vessel was put into an oven at 200 ºC for 2 h. Then, the CNTs were recovered, rinsed with water until neutrality, and dried overnight at 120 ºC. The resulting materials were labelled as CNTox-Y, where Y corresponds to the HNO_3 concentration (in M) used in the oxidation treatment (Y = 0.050, 0.10, 0.20 and 0.30). These materials were used for the subsequent chemical functionalization.

2.2.2 Silanization and addition of cross-linkers

For the treatment with glutaraldehyde (crosslinker), 4.0 mg of CNTox-Y samples were added to 0.75 mL of a 5% glutaraldehyde solution in 50 mM phosphate buffer (pH 7.0). The suspension was stirred for 2 h at room temperature and then centrifuged and washed with buffer. Materials were labelled as CNTox-Y-G.

Silanization of CNTox-Y surface was performed by adding 0.1 g of CNTox to 15 mL of a 1% aqueous solution of APTES. The suspension was left under stirring for 24 h at 50 ºC. After this step, the nanotubes were filtered, washed with water, and dried overnight at 100 ºC. The resulting materials were designated as CNTox-Y-A.

A combination of the two above-mentioned treatments was also performed, i.e. silanization and then addition of a crosslinker (CNTox-Y-AG samples).

Finally, the treatment of CNTox-Y with EDC (as crosslinker) and NHS (as stabilizer) was also performed and materials were labeled as CNTox-Y-EN. Briefly, 4.0 mg of CNTox-Y was added to 1.5 mL of a buffer solution (50 mM phosphate buffer; pH 8.0) containing 40 mg L^{-1} of NHS and 20 mg L^{-1} of EDC. The suspension was stirred for 1 h. Then, the modified CNTs were centrifuged and washed twice with the buffer solution.

2.3 Preparation of polysulfone membranes

Polyssulfone (PSf) membranes blended with CNTs oxidized with 0.3 M HNO_3 were prepared by a non-solvent induced phase separation method similar to that reported elsewhere [20]. In a standard procedure, different amounts of CNTs (0.1, 0.3 and 0.5 wt.%) were dispersed in 30 mL of NMP until achieving a uniform distribution, then polyvinylpyrrolidone (PVP) 2 wt.% and PSf polymers 12 wt.% were added to the resulting NMP dispersion at 70 ºC for 2 h. After that, the dope was cooled down and casted on a glass dish through spin-coating (SPSf-Europe, SPIN 150) and subsequently, immersed into a distilled water coagulation bath at room temperature to form the corresponding membranes, which were stored in a distilled water bath until being used. A neat PSf membrane without any amount of CNTs (referred as M) was also prepared following the same experimental procedure. PSf membranes were labelled as follows:

z-CNT/M, where z is the amount of functionalized CNTs used (ranging from 0.1 to 0.5 wt.%).

2.4 Characterization techniques

The surface area of the materials was determined by N_2 adsorption–desorption at -196 ºC, measured on a Quantachrome NOVA 4200e apparatus. Samples were first degassed in vacuum for 3 h at 120 ºC before analysis. The Brunauer–Emmett–Teller (BET) specific surface area (S_{BET}) was determined from the nitrogen adsorption data within the 0.05–0.15 range of relative pressure. Fourier transform infrared (FTIR) analysis was carried out on a FT-IR Nicolet 510-P spectrometer (Thermo Fisher Scientific, USA) equipped with a MIRacle™ Single Attenuated Total Reflectance (ATR) ZnSe crystal plate accessory (PIKE Technologies, USA). Raman spectra were recorded in a Brucker RFS100/S FT-Raman spectrometer (Nd:YAG laser, 1064 nm excitation), at a power of 200 mV, with 3000 scans at a resolution of 4 cm^{-1}. A JEOL 2010F analytical electron microscope, equipped with a field-emission gun was used for transmission electron microscopy (TEM) images.

The morphology of PSf membranes was characterized by scanning electron microscopy (SEM) using a FEI Quanta 400FEG ESEM/EDAX Genesis X4M instrument. The membranes were frozen and broken under liquid nitrogen. The microscope was equipped with a special multiple sample holder, in which the broken membranes were vertically positioned to cross-sectional analysis.

2.5 Laccase immobilization over CNTs and CNTs/PSf membranes

Enzyme immobilization was carried out by adding 4.0 mg of CNTs to 1.2 mL of a 3.75 μL_{lac} mL^{-1} laccase solution in 50 mM phosphate buffer (pH 8.0), under orbital

stirring for 2 h at 25 ºC. The pH of immobilization (pH 8.0) was chosen based in preliminary studies on the effect of this parameter in the activity of free laccase (see Supplementary Information, Fig. SI1). For the materials treated with APTES and glutaraldehyde (CNTox-Y-AG), the immobilization time and temperature were set at 24 h and 4 ºC, respectively. The activity of the laccase stock solution, determined by using ABTS as substrate, was 1600 U mL^{-1}. After immobilization, CNTs were washed several times with the appropriated buffer. Control tests for enzyme leaching were carried out by measuring the enzyme activity in the buffer after washing CNTs. No enzyme activity was detected for all materials used. Immobilization yield (%) is defined as the difference in activity of the free enzyme in solution and the activity of the free laccase remaining in the supernatant after immobilization, divided by the activity of the enzyme in the free form times 100%. Immobilization of laccase over CNTs/PSf membranes was carried out by immersing the prepared membranes (area of 5 cm^2) in 20 mL of laccase solution (buffered with phosphate, pH 8.0) for 1h at 25 ºC. Finally, the membranes were washed 3 times with the same phosphate buffer.

2.6 Enzyme activity tests for free and immobilized laccase

Free laccase activity was determined spectrophotometrically (JASCO V-560 UV–Vis spectrophotometer) for the oxidation of 0.4 mM ABTS substrate in (0.05 mM citrate/0.1 mM-phosphate-buffer) at pH 4.5. The activity was measured by incubating 0.1 mL of the enzyme solution mixed with 1.9 mL of the ABTS solution (to a 2.0 mL total volume) at 40 ºC [34]. ABTS oxidation was followed by measuring the absorbance at 420 nm (ε_{420nm}=36000 M^{-1} cm^{-1}) during time. The catalytic activity was obtained from the slope of the initial linear portion of the kinetic curve. The data (absorbance versus time) was automatically measured using the kinetic mode by the

spectrophotometer (POR AQUI O MODELO, ETC.). One unit (U) was defined as the amount of enzyme required to oxidize 1 μmol of ABTS min^{-1}. For the free enzyme system, the activities were expressed in U L^{-1}.

To measure laccase activity when using immobilized enzyme, the modified CNTs or CNTs/PSf membranes were mixed with 105 mL of citrate/phosphate buffer 0.05 M/0.1 M, pH 4.5 at 40 ºC and 37.5 mL of ABTS 0.4 mM, under magnetic stirring at 100 rpm. Samples were filtered with 0.45 μm polypropylene filters with exception of CNTs/PSf membranes, where filtration was not needed. The enzyme activity was determined using the following expression:

$$\frac{U}{g} = \frac{A_{min} \times f_{dil} \times V_{rxn} 10^6}{\varepsilon_{420nm} \times m_{CNT}} \quad (1)$$

where, U/g is the amount of enzyme capable to oxidize 1 μmol of ABTS (per minute and per mass unit of CNTs); A_{min} is the absorbance per minute (determined by linear regression); f_{dil} is the dilution factor; V_{rxn} is the volume of reaction (in mL); 10^6 is the conversion factor of M into μM; ε_{420nm} is the ABTS molar absorption coefficient; and m_{CNT} is the amount of CNTs, either in powder form or immobilized into membranes (in grams of CNT in the membrane, not the total mass of membrane).

2.7 Thermal stability of free and immobilized laccase

Thermal stability studies were performed by incubating the free and immobilized enzyme in phosphate buffer (100 mM, pH 8.0) at different temperatures (40-60 ºC). Samples of the enzyme solution and of CNTs-laccase bioconjugate were kept in a temperature-controlled water bath. A sample was taken regularly, and the

enzymatic activity was immediately determined according to the above-described methods.

The thermal parameters were determined following a simplified deactivation model [35, 36]:

$$E \overset{k_1}{\rightarrow} E_1 \overset{k_2}{\rightarrow} E_2 \tag{2}$$

$$A = \left[100 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right] e^{-k_1 t} + \left[\frac{\alpha_2 k_1}{k_2 - k_1} - \frac{\alpha_1 k_1}{k_2 - k_1}\right] e^{-k_2 t} + \alpha_2 \tag{3}$$

where A is the relative residual enzyme activity, α_1 and α_2 are the ratios of specific activities (remaining activities) to the different states E_1/E and E_2/E (see Eq. 2), respectively, k_1 and k_2 are the thermal inactivation rate constants and t is the incubation time. The experimental results suggest that laccase undergoes a conformational transition when submitted to high temperatures, the inactivation following a single exponential decay, in which $\alpha_2 = 0$ and $k_2 = 0$:

$$A = (100 - \alpha)e^{-kt} + \alpha \tag{4}$$

The thermal parameters α and k were determined by non-linear fitting over the experimental data. The biocatalyst half-life ($t_{1/2}$) was obtained from Eq. 4, using the estimated parameters (k and α) and taking A as one-half of A_0, the initial residual enzyme activity.

2.8 Operational stability of immobilized laccase

To investigate the reuse of the immobilized enzyme, the CNTs-laccase bioconjugates were tested for ABTS oxidation and then, the material was recovered by filtration and thoroughly washed with buffer solution. Then, it was added to a fresh substrate solution, and the biocatalytic activity determined. The activity of the immobilized enzyme after the first reaction cycle was used as reference (corresponding to 100%) and the activities obtained in the 4 subsequent cycles were compared to that value.

2.9 Biocatalytic degradation of phenolic compounds by laccase

The degradation of a mixture of phenolic compounds containing phenol, resorcinol, 4-methoxyphenol and 4-chlorophenol was evaluated using free laccase and the enzyme immobilized into CNT-0.30-EN. In these reactions ABTS was used as mediator. The reaction solution (20 mL), containing a mixture of the phenolic compounds (with a concentration of 10 mg L^{-1} in each component), laccase 1 μL mL^{-1} (free enzyme) or 1 mg mL^{-1} (immobilized enzyme) and ABTS (0.1 mM), was magnetically stirred for 60 min. A similar procedure was adopted for the reactions using the enzyme immobilized over CNTs/PSf membranes. In this case, the biocatalytic tests were performed for the degradation of 4-methoxyphenol (MP) in the absence of any mediator. Reactions were carried out in triplicate and data are expressed as the average of three independent assays.

Samples were periodically withdrawn, and the concentration of each compound was determined by high performance liquid chromatography (HPLC) using a Hitachi Elite LaChrom apparatus equipped with L-2450 diode array detector and a Purospher Star RP-18 endcapped column (250 mm × 4.6 mm, 5 μm particles) working at room temperature. The mobile phase consisted in a mixture of water (W): methanol (M), with

proportions varying from (70:30) to (47:53) in 16 min at a flow rate of 1 mL min^{-1}. The initial conditions were reset in a 1 min gradient step and the W:M (70:30) mixture was eluted for 6 min before starting the next analysis.

3. Results and discussion

3.1 Materials characterization

Surface interactions between the enzyme and the support were investigated by vibrational spectroscopy, carrying out FTIR-ATR analyses of selected materials, namely pristine CNTs, CNTox-0.30, CNTox-0.30-EN and laccase immobilized over CNTox-0.30-EN. As expected, the vibrational structure of the surface is significantly changed as new bands appear in the infrared spectrum after oxidation of CNTs with HNO_3. The intensity of the new occurring bands increases with the concentration of acid solution used in the liquid phase treatment (Fig. 1a). Four main groups of bands can be observed in the FTIR spectrum of CNTox-0.30 (Fig. 1a) attributed to oxygenated groups: i) a band at 1800 cm^{-1} attributed to the C=O stretching vibration of carboxylic acids ii) a peak at 1630 cm^{-1}, due to vibration of O−H bonds of adsorbed water [37]; iii) a broad band centered at 1400 cm^{-1} assigned to O−H bending in phenols and carboxylic acids; iv) a set of bands in the region 1200-1020 cm^{-1} assigned to the C−O stretching vibration in phenols. An additional band peaking at 880 cm^{-1} can be ascribed to isolated aromatic C−H out-of-plane bending vibrations.

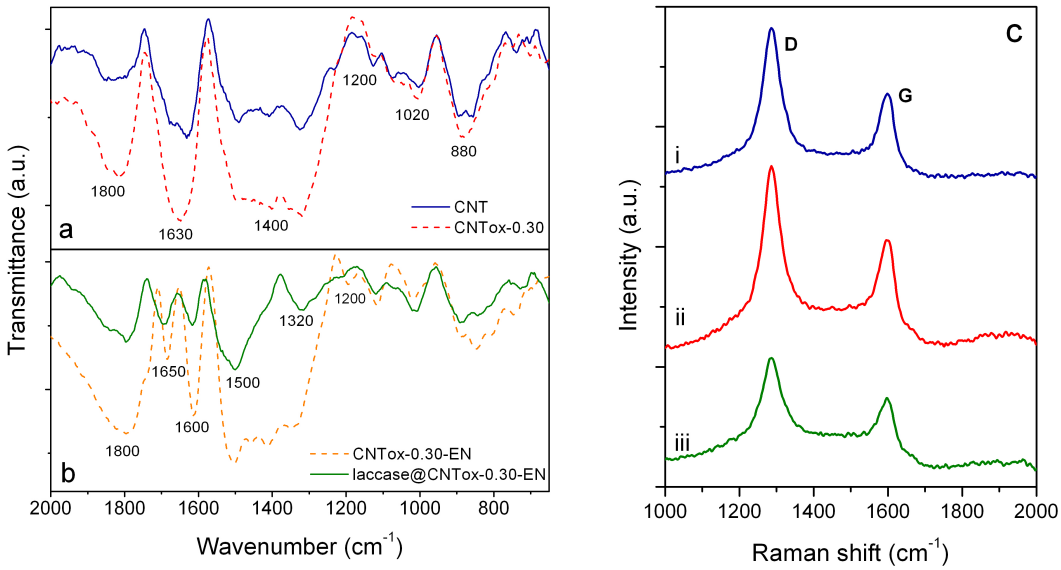


Figure 1. FTIR-ATR spectra of: (a) CNT and CNTox-0.30; (b) CNTox-0.30-EN and laccase immobilized on CNTox-0.30-EN. (c) Raman spectra of pristine CNT (i), CNTox-0.30-EN (ii) and laccase immobilized on CNTox-0.30-EN (iii).

After treatment with EDC/NHS the 1630 cm^{-1} band splits in two new bands, at 1600 cm^{-1} and 1650 cm^{-1}, respectively (Fig. 1b). These bands are related to peptide C=O stretch and peptide N−H bend, respectively, typical from the amide signature in the infrared spectra [38]. The band at c.a. 1200 cm^{-1} refers to C−N vibration of amide group. In addition, an increase in the intensity of the band at 1800 cm^{-1} indicates the attachment of the succinimidyl ester (–COOsuc) termination on the material surface. This intense band is the result of the overlap of different peaks, namely at 1820 cm^{-1} related to –COOsuc carbonyl stretch, at 1785 cm^{-1}, attributed to the suc-cycle C=O symmetric stretch and at 1745 cm^{-1} corresponding to suc-cycle C=O antisymmetric stretch, as previously described in the literature [38]. These results confirm the successful functionalization of CNTox-0.30 with EDC/NHS.

After laccase immobilization, a decrease in the intensity of the bands at 1600 cm^{-1}, 1650 cm^{-1} and at 1200 cm^{-1} suggests that the enzyme bounds to amide terminal groups of the CNTs functionalized with EDC/NHS (Fig. 1b). Furthermore, the appearance of two new bands, one at 1320 cm^{-1}, due to C−N stretching vibration of amines, and another at 1500 cm^{-1}, ascribed to ring stretching vibration of side chain aromatic amino acids, is indicative of the presence of laccase on the surface of CNTox-0.30-EN [9, 39].

The Raman spectra of pristine CNTs, CNTox-0.30-EN and laccase modified CNTox-0.30-EN (Fig. 1c) exhibit two characteristic main bands in the 1000-2000 cm^{-1} region. The first order G mode peaking at c.a. 1600 cm^{-1} is ascribed to the regular sp^2 graphitic network of CNTs, while the D mode band at 1287 cm^{-1} results from the disorder and defects in the carbon lattice [40]. The ratio between the intensities of D and G bands (I_D/I_G) may be used to assess the degree of purity and the existence of defect sites as well as the extent of CNTs functionalization [41]. The I_D/I_G values observed for pristine CNTs, CNTox-0.30-EN and laccase modified CNTox-0.30-EN were 1.55, 1.66 and 2.81, respectively. This increase in the I_D/I_G ratio upon functionalization of CNTs with the cross linker and the enzyme indicates an increase in the disorder of the CNTs' surface due to the covalent immobilization of laccase [42]. The morphological analysis of PSf membranes blended with functionalized CNTs (i.e., CNT/M) and the neat PSf membrane (M) was carried out by SEM. Fig. 2 shows representative images of the top surfaces and cross-sections at different magnifications. In general, all membranes presented an asymmetric structure with a similar dense top surface and a porous sub-layer. The incorporation of different amounts of functionalized CNTs did not influence significantly the morphology of the membrane surface (Figs. 2a, d and g), but a more porous structure with elongated finger-like pores

was obtained observed across the CNT/M membranes regardless the amount of functionalized CNTs, according the cross-section micrographs (Figs. 2b-c *vs.* e-f and g-i). In addition, functionalized CNTs were homogenously dispersed throughout the polymer structure even at high loadings (as confirmed by visual analysis, see Supplementary Information, Fig. SI2), although some isolated CNT bundles were also found through the finger-like pores of the membranes (Fig. 2i for 0.5-CNT/M). These results suggest that the chemical functionalization of CNTs improved a fast solvent/non-solvent exchange during phase inversion of the membranes, favoring the formation of this type of pores [20].

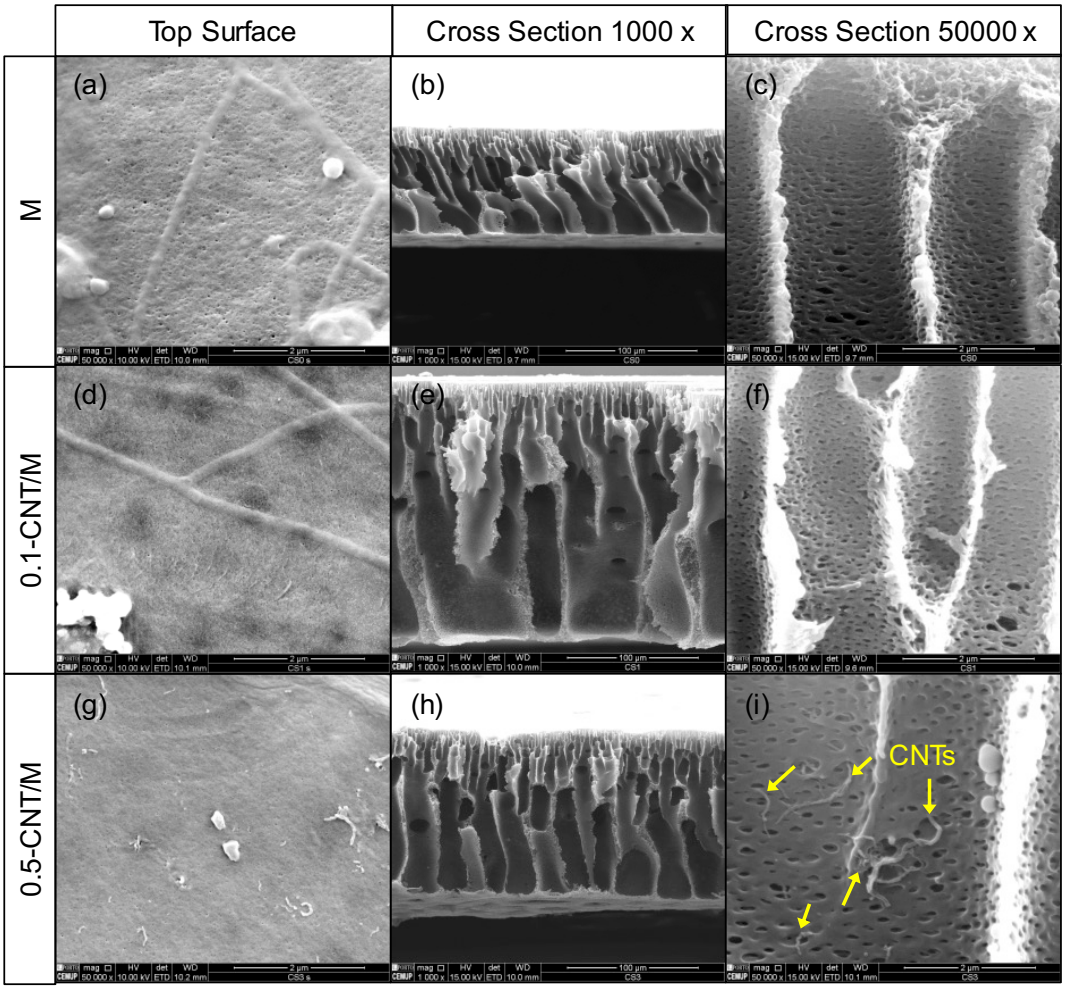


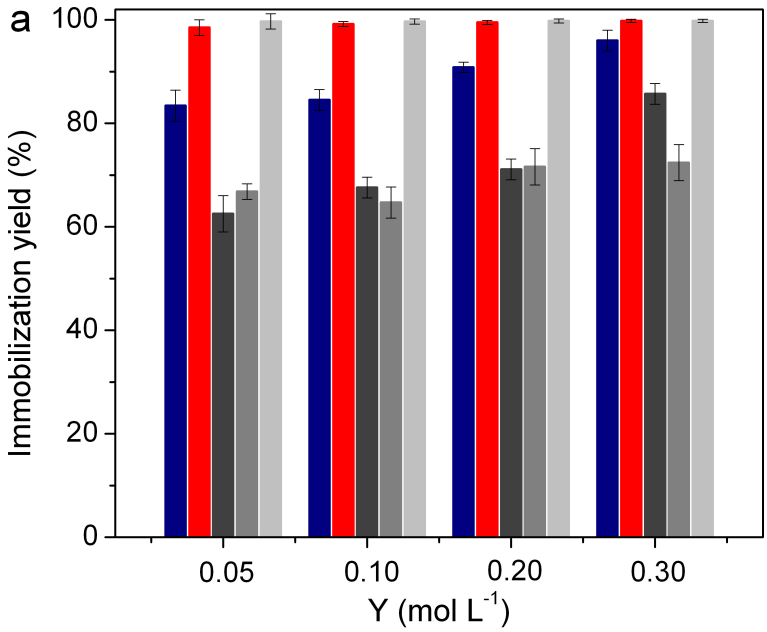
Figure 2. SEM images of the (a, d and g) top surface and (b-c, e-f, and h-i) cross-section of the neat PSf membrane (a-c) and functionalized CNT/M membranes (d-i).

3.2 Laccase immobilization and catalytic activity on functionalized CNTs

The CNTs have proved their capacity to act as carriers of a great variety of compounds and biomolecules including enzymes [43]. Adsorption of enzymes into CNTs occurs through different types of processes including electrostatic or hydrophobic interactions [44], hydrogen bonding and π-π interactions [43]. The nature and intensity of such interactions depends on both the enzyme's structure and the surface chemistry of the CNTs. Liquid phase oxidation using HNO_3 is known as an efficient method for introducing oxygen groups at the surface of carbon materials [9, 45, 46]. Depending on the concentration of the acid, different amounts of oxygen-containing groups such as carboxylic acids, phenols, quinones and carbonyl groups, can be introduced. In this work, CNTs were oxidized with HNO_3 concentrations ranging from 0.05 to 0.30 M (CNTox-Y).

The laccase immobilization yield over the functionalized CNTs was found to increase with Y, reaching a maximum value of 96% for CNTox-0.30 (Fig. 3a). Carboxylic acids are expected to be the predominant functionalities introduced at the surface of CNTs throughout oxidation with HNO_3 [46, 47]. Therefore, an increase in the acidity of CNT surface and a decrease of the pH_{PZC} to very low values is expected upon treatment of the carbon materials with increasing HNO_3 concentration. Since the immobilization of the enzyme was performed at pH 8.0, the surface of the CNTox-Y materials is expected to be negatively charged. Moreover, as the isoeletric point of laccase occurs at pH 4.2 [48], the molecule is also negatively charged during the immobilization step. Thus, electrostatic interactions are not likely to be the main driving

force for the immobilization of laccase into CNTox under the used conditions. In this case, laccase immobilization may be attributed to hydrophobic and π–π stacking interactions between the enzyme and the carbon substrate. In addition, the treatment with HNO_3 is known to create defects in the walls and to open up the end caps of the CNTs, leading to an increase in the porosity [49]. In fact, a progressive increase in the S_{BET} of CNTox was observed when treating the original CNTs with increasing concentrations of HNO_3 (S_{BET} = 73 m^2g^{-1} and 83 m^2g^{-1} for pristine CNT and CNTox-0.30, respectively), leading to a higher adsorption capacity of the treated CNTs (14% increase, in this case).



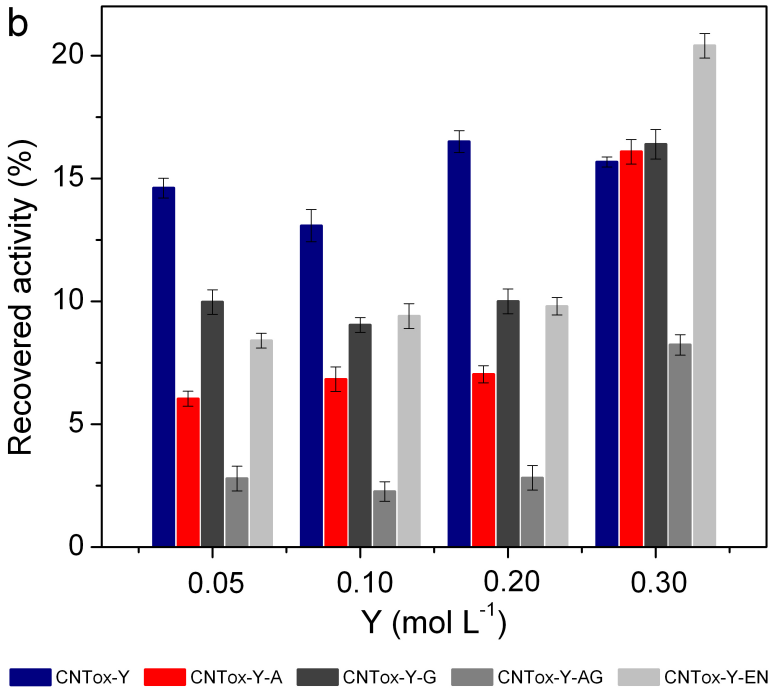


Figure 3. Laccase immobilization efficiency (a), and recovered activity (b), as a function of HNO_3 concentration (Y) for surface modified CNTs. Experimental conditions: laccase immobilized in 50 mM phosphate buffer (pH 8.0) under orbital stirring for 2 h at 25 ºC.

The silanization of CNTox-Y was performed by treating the materials with APTES. In this case, the OH groups present at the surface of CNTox act as anchoring spots for APTES. This procedure resulted in a laccase immobilization yield above 98% using all CNTox-Y-A materials regardless the HNO_3 concentration used for oxidizing CNTs (Fig. 3a). This result can be rationalized by the simultaneous action of APTES as anchoring agent and also as spacer between the surface of neighbor CNTs, allowing steric freedom to the biomolecule during the immobilization step [50]. In addition, direct laccase adsorption on the surface of CNTox-Y may also occur, contributing for this improvement in the immobilization yield.

Glutaraldehyde activation of supports is one of the most common techniques for immobilizing enzymes [51-53]. In this method, the enzyme is immobilized mostly

through the amine groups either ε-amine groups of lysine residues or terminal groups [54]. In the present work, the CNTox-Y materials treated with glutaraldehyde, resulted in CNTox-Y-G materials displaying a lower immobilization capacity compared to the parent. For example, it was observed a decrease in laccase immobilization yield from 96.0% to 85.7% by comparing the performance of CNTox-0.30 to CNTox-0.30-G. A possible reason for this decrease is the blockage of CNTox-Y surface by glutaraldehyde molecules. Similar immobilization yield was obtained for CNTox-Y-G materials with Y between 0.05 and 0.10 mol L^{-1}, and a relatively higher efficiency was observed for the material treated with HNO_3 0.30 mol L^{-1}, probably due to a higher amount of glutaraldehyde present at the surface of CNTox-Y-0.30.

Another technique used for improving the immobilization of enzymes into solid carriers is the combination of APTES with glutaraldehyde. In this case, the previously prepared CNTox-Y-A materials, which have free amino-groups on their surface, were post-treated with glutaraldehyde yielding CNTox-Y-AG. In this case coupling glutaraldehyde to CNTox-Y-A didn't provide any positive effect in the immobilization of laccase (Fig. 3a), which may be ascribed to the obstruction of part of laccase anchorage centers by glutaraldehyde molecules. Here, it is expected that most of the enzyme may be covalently bonded to CNTs. The highest immobilization yield (72.4%) using this series of materials was obtained using CNTox-0.3-AG.

Finally, the covalent immobilization of laccase into CNTs was attempted by the modifying the surface of the carbon materials with EDC and NHS (CNTox-Y-EN). EDC is a zero-length cross-linker widely used in protein conjugations. EDC reacts with a carboxylic acid group at the surface of the support, forming an amine-reactive *O*-acylisourea intermediate which subsequently reacts with an amine group of the enzyme to produce a stable amide bond. Yet, this intermediate is highly unstable and can be

easily hydrolyzed. NHS is normally used as stabilizer, converting the *O*-acylisourea intermediate into an amine-reactive ester, thus increasing the coupling efficiency [55, 56]. CNTox-Y-EN materials show excellent capacity (c.a. 100%) for laccase immobilization, regardless the HNO_3 concentration used for oxidizing CNTs (Fig. 3a). In terms of recovered activity, the best results (15.0%) were in general obtained for the CNTox-Y materials in which the enzyme is immobilized by direct adsorption (Fig. 3b). For this series of materials, although the enzyme may be more active, the enzyme-support bond is expected to be weaker, which may be a drawback in what concerns reutilization. In spite of total enzyme immobilization achieved with CNTox-Y-A, the recovered activity was in general low, especially for Y between 0.05 and 0.20. Despite the lower amount of laccase immobilized into CNTox-Y-G, the recovered activity was slightly higher than that achieved using the analogous CNTox-Y-A materials.

The activity of laccase immobilized into CNTox-Y-AG was in general very poor. The best result was obtained for the material prepared with the CNTs oxidized with the 0.30 M HNO_3 concentration (CNTox-0.30-AG), resulting in merely 8.3% of recovered activity. As the recovered activity was so low, laccase immobilized into CNTox-Y-AG was discarded for further studies. Besides the high immobilization yield, multiple attachments with groups from the active site of the enzyme may lead to a loss of activity.

In general, the best results in terms of recovered activity were obtained using laccase immobilized in CNTox-0.30, which strengthens the importance of the oxidation of the surface of CNTs for an improved efficiency as laccase carrier. Among all the materials tested for laccase immobilization, the highest value of recovered activity (20.5%) was obtained with CNTox-0.30-EN. In this case it is expected that most of the

enzyme is covalently bonded to the support, as suggested by FTIR-ATR analysis (Fig 1b), improving its stability and catalytic performance.

3.3 Thermal stability of free and immobilized laccase

It is generally accepted that the immobilization of enzymes may provide a protecting effect against enzyme deactivation following temperature increase. The immobilization process could affect the conformational flexibility of enzymes, resulting in an increase in enzyme rigidity. Submitting enzymes to progressively increasing temperature is one of the strategies for evaluating the changes in protein structure and enzyme stability towards denaturation resulting from heating [57]. Enzymes, such as laccase, are sensitive to temperature, which affects not only its own conformation but also the enzyme-substrate interaction, and consequently the catalytic performance at elevated temperatures [2]. The thermal stability of laccase immobilized on CNTox-0.30 was evaluated and compared with the performance of the free enzyme, by running tests at three different temperatures, namely 40, 50 and 60 ºC (Fig. 4).

The experimental data followed an exponential decay model (Eq. 4). In this model, it is considered that the enzyme inactivation takes place in only one stage (the one-step transition between the active and denatured state) with the possibility of existing a remaining activity, represented by the parameter α.

As expected, the increase in temperature leads to a decrease in the stability of the enzyme and reduction of its activity (Fig. 4). This inactivation is attributed to the increase in the vibration of the laccase structure when heated, that may lead to the breaking of chemical bonds and ultimately to a change in its 3D structure [9].

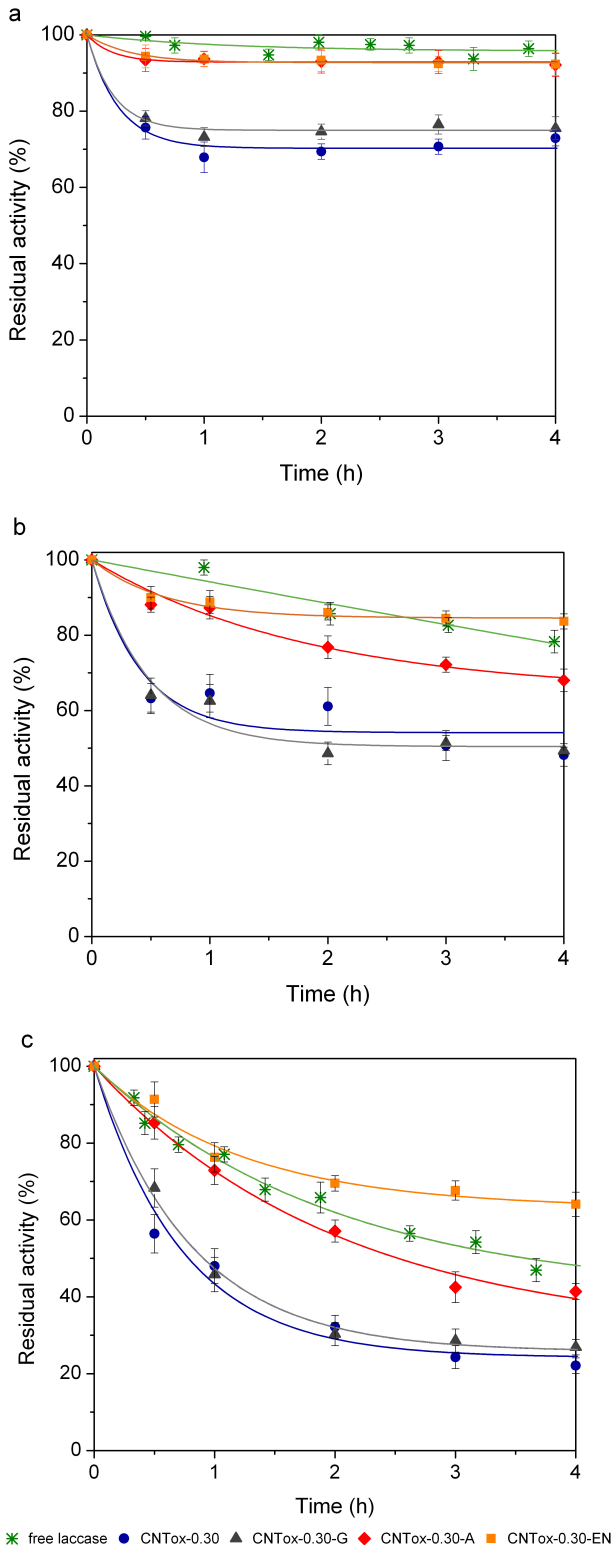


Figure 4. Thermal inactivation of free and immobilized laccase, measured in terms of residual activity (%) over a period of 4 h, in phosphate buffer (100 mM, pH 8.0) at temperatures of: (a) 40 ºC; (b) 50 C; and (c) 60 ºC.

Overall, the most thermally stable system was the one based on CNTox-0.30-EN. For laccase immobilized in this material and incubated at 40 ºC, it can be observed that after a first rapid decay in the enzyme activity, the performance remained nearly constant (residual activity of c.a. 93%). As the temperature increases, the residual activity decreases, being of 84% and 71% after 4 h of incubation for the tests performed at 50 and 60 °C, respectively.

A good thermal stability was also obtained for the material functionalized with APTES. The thermal stability at 40 °C is similar to laccase immobilized over CNTox-0.30-EN, but is relatively lower for the higher temperatures. These results suggest that the functionalization of CNTs with EDC/NHS and with APTES increase the stability of laccase when subjected to high temperature (between 40 and 60 ºC) which may be attributed to a strong interaction between the enzyme and the support preventing thermal denaturation. It is worth noting, that at 60 ºC, the parameter α (remaining enzyme activity) is almost 2 times higher for CNTox-0.30-EN when compared with the other systems, confirming the enhanced performance of this material for laccase immobilization (Table 1).

Regarding the kinetics of laccase thermal inactivation, quantified by the kinetic rate constant (k), it was observed that the initial inactivation of the bioconjugates was faster for lower temperatures. For instance, in the case of laccase immobilized over CNTox-0.30-EN, a kinetic deactivation constant of 2.71 h^{-1} was obtained for the reaction at 40 ºC, while the value decreases to 1.64 and 0.82 h^{-1} when the system was submitted a temperature of 50 and 60 ºC, respectively. Yet, after this initial inactivation phase, the enzymes retained their activity, as confirmed by the higher α values. In fact, the half-life ($t_{1/2}$) could not be determined at 40 and 50 ºC (except for free laccase at 50 ºC) since the residual activity was kept above 50% during the biocatalytic runs (Figs.

4a-b). The same was observed when laccase was immobilized over CNTox-0.3-EN (Fig. 4c).

Table 1. Thermal stability kinetic parameters of free and immobilized laccase in phosphate buffer (100 mM, pH 8.0) at different temperatures over a period of 4 h.

Temperature	Thermal parameters								
	40 ºC			50 ºC			60 ºC		
	α (%)	k (h^{-1})	$t_{1/2}$ (h)	α (%)	k (h^{-1})	$t_{1/2}$ (h)	α (%)	k (h^{-1})	$t_{1/2}$ (h)
Free laccase	-	-	[a]	32.0	0.09	14.4	26.5	0.36	3.16
CNTox-0.3	70.4	3.89	[a]	54.1	2.44	[a]	28.0	1.38	0.86
CNTox-0.3-G	70.9	5.20	[a]	50.4	2.10	[a]	25.8	1.23	0.91
CNTox-0.3-A	92.9	4.73	[a]	64.6	0.54	[a]	28.9	0.48	2.54
CNTox-0.3-EN	92.7	2.71	[a]	84.5	1.64	[a]	63.0	0.82	[a]

[a] residual activity was kept above 50%

The increase in thermal stability of laccase when the enzyme was immobilized over CNTox-0.30-EN may be attributed to the reduction in the enzyme structure mobility, due to its anchorage at the surface of the support [58].

3.4 Operational stability of immobilized laccase

One of the most important advantages of enzyme immobilization is the possibility to recover and reuse it in repeated catalytic cycles. The reutilization tests (Fig. 5) reveal that after 5 cycles of utilization, the highest activity was obtained for CNTox-0.30-EN. After 5 cycles, the enzyme immobilized on this material remained with 66% of its initial activity, contrasting with 38%, 29% and 18% obtained using CNTox-0.30-A, CNTox-0.30 and CNTox-0.30-G, respectively.

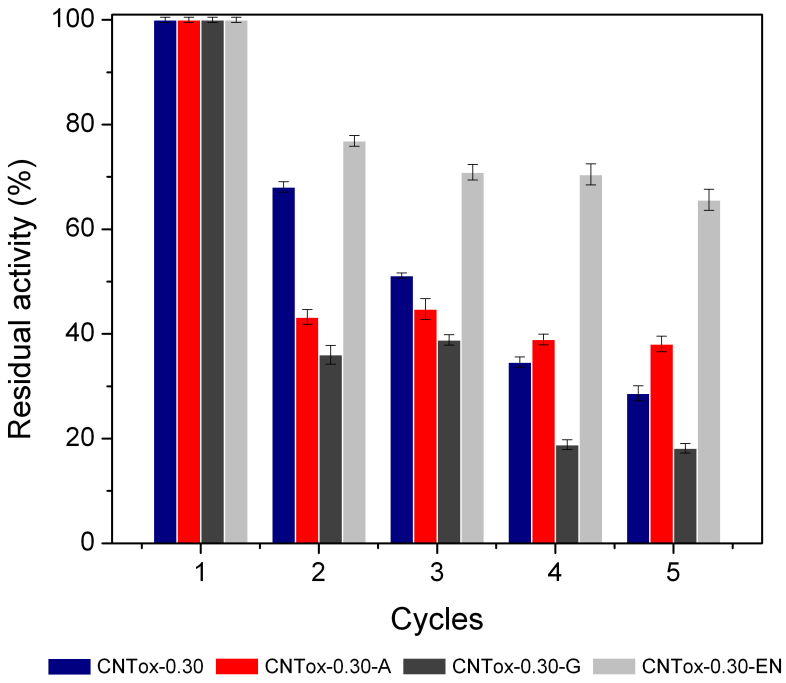


Figure 5. Residual immobilized laccase activity in 5 consecutive cycles of catalytic reaction. Experimental conditions: laccase immobilized in 50 mM phosphate buffer (pH 8.0) under orbital stirring for 2 h at 25 ºC.

The high activity loss of CNTox-0.30-G at the end of the first cycle (c.a. 64%), is in line with the results obtained in the thermal stability study. The residual activity obtained using laccase immobilized over CNTox-0.30 show a steady decline over the five cycles. Nevertheless, at the end of the first and second cycles, the enzymatic activity was higher comparing to CNTox-0.30-G and CNTox-0.30-A. For CNTox-0.30-A, although there is a high activity loss immediately after the first reuse (57%), the enzyme activity remained nearly constant in the range 38-44% over the five cycles of reaction.

3.5 Degradation of phenolic compounds using CNT/laccase bioconjugates

Phenolic compounds may be found in variety effluents discharged from industrial and agricultural sources including olive oil and pulp mill wastewaters, wastes

from coal conversion, manufacture of resins, plastics, adhesives, leather, among others. Conventional wastewater methods for treating phenolic effluents include biological treatment, adsorption, chemical oxidation such as ozonation and Fenton reaction, and photocatalytic processes [59]. However, these techniques are costly, time consuming, require metals, other chemicals, and may be inefficient for the removal of phenol. On the other hand, enzymatic wastewater treatment can be very efficient, due to the high degree of specificity of enzymes and because of the minimal environmental impact [60]. The best performing system, CNTox-0.30-EN, was selected for the treatment of an aqueous mixture containing four phenolic compounds normally used as model molecules because they are often present in many different effluents: phenol (PH), resorcinol (RS), 4-methoxyphenol (MP) and 4-chlorophenol (CP) [61, 62].

The degradation of the phenolic compounds was firstly carried out using free laccase, both in the presence and absence of ABTS as enzyme mediator (Figs. 6a and b).

The parent PH is quite refractory to the action of the enzyme, even in the presence of ABTS, with less than 10% being removed at the end of 60 min of reaction. RS was hardly degraded by laccase in the absence of the mediator. Nevertheless, it was almost totally degraded by the laccase mediator system (94% removal) after 1 h. In the case of CP, the presence of ABTS led to an increase in degradation from 22 to 41%. MP was the most easily degraded compound with total removal being achieved at the end of the reaction even in the absence of ABTS and at the end of merely 5 min using the laccase mediator.

The enhanced catalytic efficiency of laccase in the presence of ABTS results from the mediating role of ABTS which acts as an “electron shuttle”, *i.e.* the oxidized ABTS diffuses away from the enzyme and oxidizes substrate by the removal of one

electron from the substrate [63]. The rate of electron transfer from the substrate to the T1 copper in the active site of laccase is usually considered as the rate-limiting step, depending on the ΔE^0 between the T1 copper and the substrate [64, 65]. Moreover, because all the phenolic compounds investigated behave as substrates for laccase, competitive effects may arise when treating the multi-component solution, resulting in a poor removal of each compound individually. In fact, a control experiment was performed using laccase/ABTS system for the degradation of phenol in aqueous medium and 90% removal of this compound was achieved at the end of 60 min of reaction (see Supplementary Information. Fig. SI3), contrasting with the ca. 10% during the catalytic treatment of the mixture of phenolic compounds (Fig. 6b).

The molecules structure and redox potential of laccase (500 – 800 mV) strongly influences the power to oxidize each of the compounds in the mixture [66]. In fact, MP, which has the lowest redox potential (540 mV), was the easiest to oxidize, while PH and CP with redox potentials of c.a. 800 mV were the most refractory ones [67].

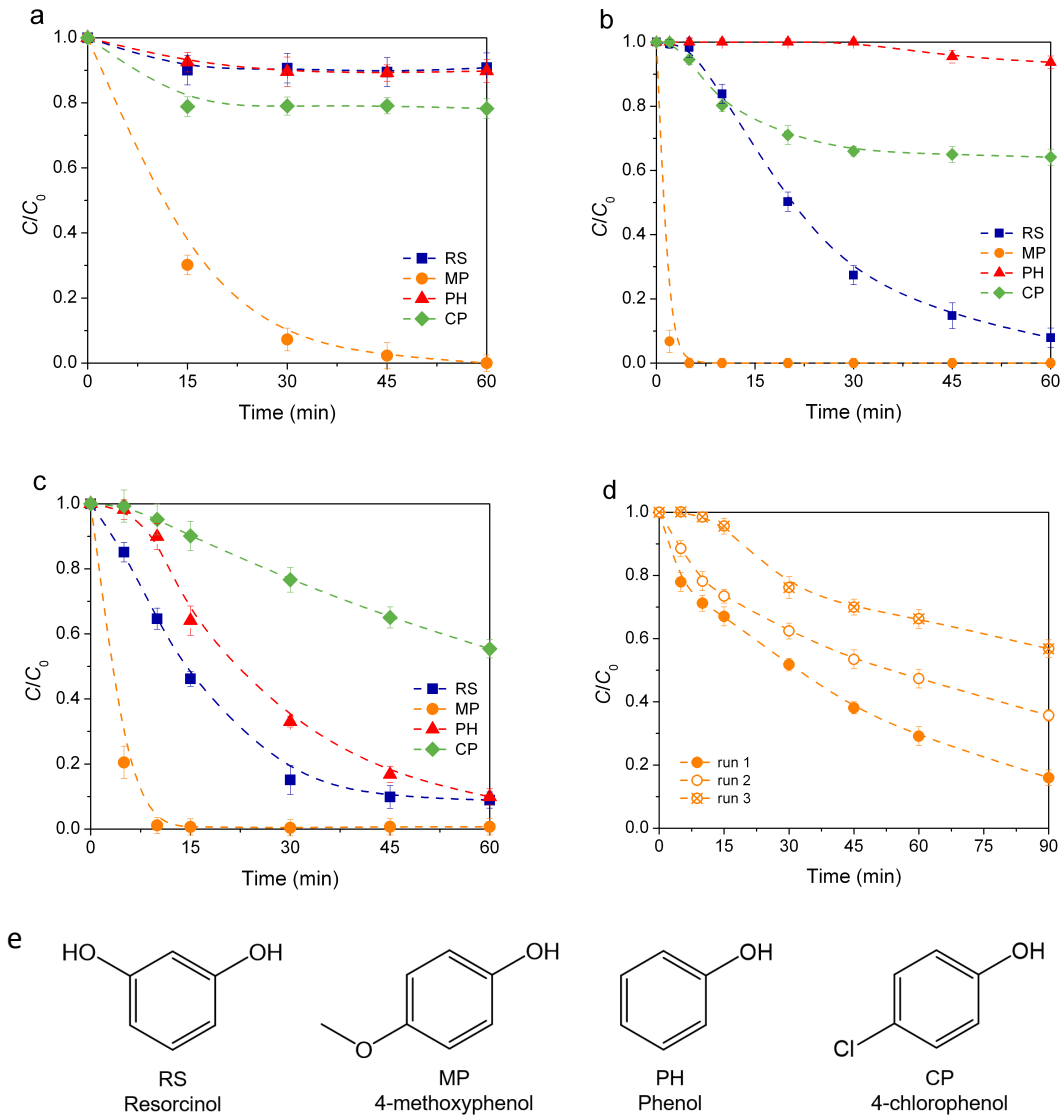


Figure 6. Degradation of a mixture of phenolic compounds over 60 min runs in the presence of: (a) free laccase with no mediator; (b) free laccase in the presence of ABTS as mediator; (c) laccase immobilized over CNTox-0.30-EN in the presence of ABTS as mediator; (d) utilization of laccase/CNTox-0.30-EN in 3 consecutive cycles for the degradation of MP with no mediator over 90 min; and (e) chemical structures of PH - phenol; RS – resorcinol; MP - 4-methoxyphenol and CP - 4-chlorophenol. Experimental conditions: reactions using 20 mL of aqueous solutions containing 10 mg L^{-1} MP or a mixture of phenolics with a concentration of 10 mg L^{-1} in each component, at natural pH (5.6).

Regarding the reaction using laccase immobilized over CNTox-0.30-EN and ABTS as mediator, a different behavior was observed. Starting with the parent PH, a 90% removal was achieved (from the mixture of phenols) at the end of 60 min of reaction contrasting with the 10% registered using the free enzyme.

A control experiment to assess the possibility of adsorption was performed by suspending CNTox-0.30-EN in the mixture of phenolic compounds (see Supplementary Supporting Information, Fig. SI4). Typically, the adsorption of phenol derivatives over CNTs is not significant, the degree of such being dependent on the functionalization of the surface. The removal of each phenolic compound over CNTs at the end of 60 min of contact followed the order: CP (17%), MP (10%), RS (5%) and PH (4%). These results indicate that adsorption is not likely to be the main active mechanism, showing how crucial the presence of enzyme is for the degradation process. In the biocatalytic process, MP was the most easily removed compound, with total degradation (100%) being achieved at the end of only 5 min and 10 min for free and immobilized laccase on CNTox-0.30-EN, respectively, in the presence of ABTS as enzyme mediator (Fig. 6b and 6c). The slower degradation of MP by laccase immobilized over CNTs when compared with the reaction using the free enzyme may be attributed to mass-transfer limitations, because of internal diffusion restrains since the substrate (MP) may not reach the active part of the immobilized enzyme. This behavior has been observed in other studies using immobilized enzymes [68, 69]. Yet, from both technological and economic standpoints, the use of the immobilized enzyme is always preferable due to the possibility of reutilization.

The kinetics of RS degradation using laccase in the free and immobilized forms are similar, while CP removal was slower but varying steadily during the reaction time.

Reuse tests were performed for MP removal using laccase immobilized over CNTox-0.30-EN in the absence of ABTS (Fig. 6d). As expected, MP was easily degraded in the absence of the mediator, attaining 84% of removal in 90 min of reaction. However, a progressive loss in enzyme activity was observed for the second and third utilizations of the laccase/CNTox-0.30-EN conjugate, probably due to the blockage of the active sites by MP or by degradation products.

3.6 Degradation of phenolic compounds using bio-catalytic CNT/PSf/laccase membranes

Enzymatic processes should possess cost-effectiveness and high productivity of the biocatalyst [70]. The enzyme immobilization on membranes provides a more easy, economical and fast enzyme recovery when compared to CNT powders solely. Therefore, considering the promising results obtained with the direct laccase immobilized over CNTox-0.30-EN, PSf membranes blended with different amounts of these functionalized CNTs (0.1, 0.3 and 0.5 wt.%) were developed and studied for laccase immobilization envisaging their fast and easy recovery and reuse. The immobilization of laccase was successfully achieved for all prepared CNT/M membranes regardless of the CNT-0.30-EN content (Table 2). In fact, different amounts of CNTox-0.30-EN into the membrane had a negligible effect on enzyme immobilization yield, a similar high capacity of immobilization being observed for all membranes evaluated (≅ 91±1%) and being comparable to the immobilization yield of laccase on CNTs solely. In terms of enzyme activity, the bio-catalytic performance of the membranes was affected by the CNT content (Table 2), the membrane containing 0.1 wt.% of CNTox-0.30-EN (0.1-CNT/M) presenting the highest activity (12663 $U/g_{CNT\ into\ membrane}$).

Table 2. Results of laccase immobilization on PSf membranes containing different amounts of CNTox-0.3-EN.

Membrane	Immobilization yield (%)	Laccase activity (U/g*)
0.1-CNT/M	89.8±	12663±
0.3-CNT/M	92.0±	8620±
0.5-CNT/M	92.0±	3278±

*$U/g_{CNT\ into\ membrane}$

All these bio-catalytic membranes were selected to evaluate their performance in a real application. MP was chosen since this compound was easily removed by laccase, as presented above. Thus, the degradation of MP was studied with CNT/M membranes and compared to those obtained for the neat PSf membrane (M) and also using the CNTox-0.30-EN nanomaterial. In general, MP was fast degraded in 15 min for all membranes containing functionalized CNTs (Fig. 7a), being their performance superior to that obtained for the neat PSf membrane (ca. 85 % MP conversion for 60 min). Furthermore, CNT/M membranes presented different laccase activities, being the best result obtained for the membranes containing the lowest CNT content (Table 2). Regarding MP removal, some differences can be observed during several degradation cycles. In general, all CNT/M membranes lost the MP removal efficiency after 3 cycles of reuse, while the M membrane lost that after 2 cycles (Fig 7a). The low efficiency on MP degradation observed for all membranes after consecutive cycles should be again due to some loss of enzyme or blockage of the active sites. Considering the reutilization ability of the membranes, the enzyme was re-immobilized over the spent membranes. The results presented in Fig. 7b show the potential of re-utilization of the membranes

for a new enzyme loading. Moreover, the highest capacity for MP degradation was observed for CNT/M membranes, in particular for 0.1-CNT/M, the membrane with the best performance.

Nevertheless, the possibility of reuse the enzyme can be considered a great advantage at both economic and technological standpoints.

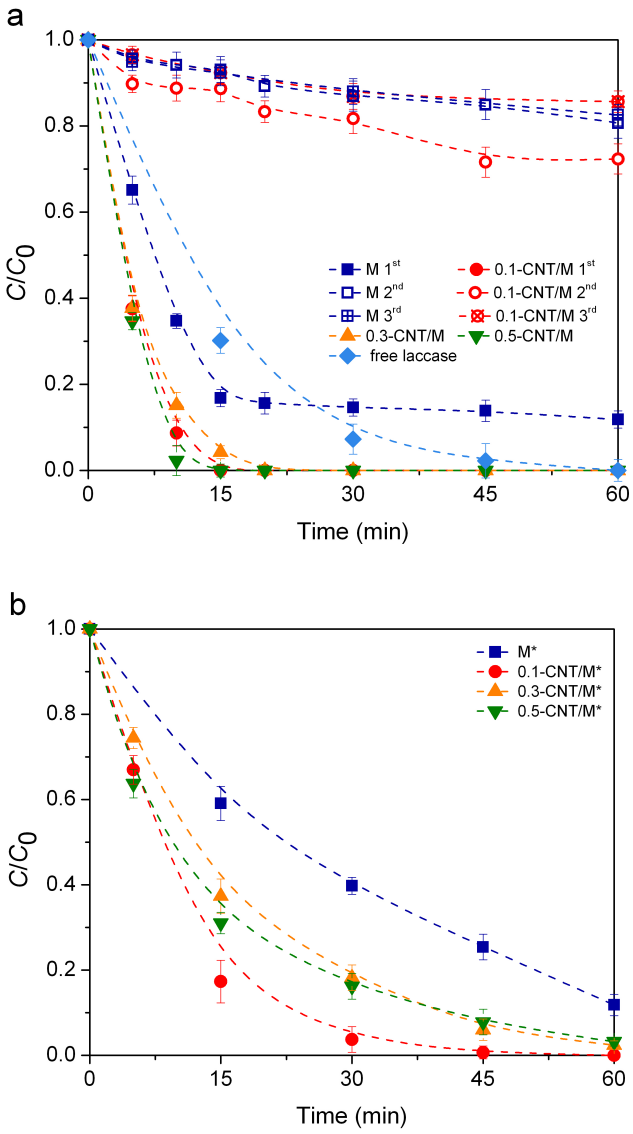


Figure 7. (a) Degradation of MP using laccase immobilized over the different PSf membranes, the 2nd and 3rd utilizations for M and 0.1-CNT/M are also included for

comparison; (b) degradation of MP using a second immobilization of laccase on used PSf membranes (M*). Experimental conditions: reactions using 20 mL of 10 mg L^{-1} MP aqueous solutions at natural pH (5.6).

In general, the properties of supported enzymes reflect not only the properties of the combined materials, but also the result of interaction between both, which can be potentially synergetic. The results here presented show not only the advantages of using a somewhat rigid material, but provide an additional indication of the strategies to tailor the material at the enzyme needs. Optimization of the functionalization of the CNTs carriers is the next step of this work. A material like CNTs provides the mechanical strength normally required to expand these applications to real industrial processes, as opposition to the well spread supporting matrixes based on soft gels.

4. Conclusions

Laccase can be efficiently immobilized over multi-walled carbon nanotubes or membranes. Higher immobilization efficiency and recovered activity was obtained using CNTs oxidized with HNO_3 0.30 M (CNTox-0.30), which is related to the higher number of oxygen-containing groups available at the surface of the material.

The system CNTox-0.30-EN possesses the best compromise between immobilization efficiency (100 %), and recovered activity (20.5%), being also the most stable material at higher temperatures.

The laccase/CNTox-0.30-EN kept its high performance in 5 consecutive utilizations for the oxidation of ABTS. The enhanced performance of CNTox-0.30-EN can be attributed to strong linkage between the enzyme and the supporting material.

The laccase/CNTox-0.30-EN bioconjugate was successfully used for the treatment of a mixture of four phenolic compounds, the process showing similar efficiency as the analogous using the free enzyme, with the possibility of reusing the biocatalyst.

PSf membranes containing functionalized CNTs demonstrated to be also an excellent support for the enzyme (re)-immobilization and their application in the degradation of 4-methoxiphenol. The PSf membrane with 0.1 wt.% of CNTox-0.30-EN was the most efficient, presenting a comparable activity to CNTs solely and with a much lower CNT content.

In summary, the results here presented validate the immobilization capacity and bio-catalytic performance of laccase on CNTs and CNT-based membranes as a technological solution in all the fields of its application. Particularly for membrane technologies, the savings on cost and the potential of combination with membrane separation functions will bring opportunities at industrial level.

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