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A novel and efficient oxidative functionalization of lignin by layer-by-layer immobilised Horseradish peroxidase

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ABSTRACT

Horseradish peroxidase (HRP) was chemically immobilised onto alumina particles and coated by polyelectrolytes layers, using the layer-by-layer technique. The reactivity of the immobilised enzyme was studied in the oxidative functionalisation of softwood milled wood and residual kraft lignins and found higher than the free enzyme. In order to investigate the chemical modifications in the lignin structure, quantitative ^{31}P NMR was used. The immobilised HRP showed a higher reactivity with respect to the native enzyme yielding extensive depolymerisation of lignin.

1. Introduction

Peroxidases are among the first enzymes to have been discovered and have been extensively studied. They catalyse the oxidation of a wide variety of organic and inorganic substrates, such as phenols, aromatic amines, thioanisoles and iodide, by means of H_2O_2.[1] Horseradish peroxidase (HRP) has been included in the class III of the plant peroxidase superfamily, composed of bacterial, fungal and plant peroxidase, and belongs to the group of heme peroxidases (EC 1.11.1.7) containing an iron protoporphyrin IX prosthetic group located at the active site.[2,3] Generally, heme-containing redox enzymes participate in a strikingly diverse range of chemistry, yet all biological oxidation reactions catalysed by these enzymes involve very similar oxidation-state intermediates.[4] The HRP catalytic cycle consists in a two one-electron oxidation steps; the active site reacts with H_2O_2 to generate a Fe(IV)-oxo radical cation intermediate, the compound I.[5,6] The first one-electron reduction step requires the participation of a reducing substrate and leads to the generation of compound II, a Fe(IV)-oxoferryl species that is one oxidising equivalent above the resting state. The second one-electron reduction step returns compound II to the resting state of the enzyme.[7]

HRP is one of the most used peroxidases due to its versatile and wide applicability in numerous and different areas such as analytical, environmental and clinical fields.[8] In fact the enzyme shows many interesting characteristics for application purposes (in reaction specificity and stability and availability in pure form at reasonable costs).[1] Improvements to these desirable qualities such as its relatively good stability in aqueous and non-aqueous solvent systems are actively sought.[9] In general the enzymes exhibit a number of features that make their use advantageous as compared to conventional catalysts; among them their chemo- regio- and stereospecificity,[10] the possibility to operate in mild reaction conditions in the frame of the development of environmentally friendly processes. However there are a number of constraints in the use of the enzymes; the common perception is that enzymes are sensitive, unstable and have to be used in water, features that are not ideal for a catalyst and undesirable in most syntheses.[11] Several approaches have been proposed to overcome these limitations; among them immobilisation is generally considered favourable for industrial scale applications since it allows for continuous processes.[12] Enzyme immobilisation can be defined as the attachment of an enzyme to a support by physical or chemical methods. Chemical immobilisation leads to the formation of irreversible covalent bonds between amino acids in enzymes and reactive groups on the support.[13] On immobilisation, enzymes are often stabilised, and thus less sensitive to denaturing agents.[11] Moreover the immobilisation provides for facile separation of the enzyme from the product, the efficient recovery and multiple reuse of the biocatalyst, plug flow processes, rapid termination of reactions and a greater variety of bioreactor design.[14] Many examples of immobilised HRP have been reported in literature.[15–18]

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The layer-by-layer (LbL) adsorption technique, introduced by Decher et al.,[19] is a general and versatile tool for the controlled fabrication of multimaterial surface coatings on a large variety of surfaces.[20] By means of this technique the construction of multilayer films is possible by the consecutive deposition of alternatively charged polyelectrolytes on a solid surface.[21] The LbL technique has been demonstrated to be an effective means for the immobilisation of enzymes.[22] In fact, polyelectrolyte capsules have the ability to protect encapsulated proteins from high-molecular-weight denaturing agents or bacteria and allow regulation of the permeability towards small substrates, which can enter and leave the protecting layers to react with the biomolecules in the interior.[23]

Today the rising energy consumption, the depletion of fossil fuel feedstocks and the increased environmental concerns have focused the attention on the use of alternative renewable materials and on the development of environmentally friendly processes that operate in mild reaction conditions. Among the most important renewable materials there is lignin, the second most abundant natural polymer on the planet. Lignin is a random three-dimensional phenylpropanoid polymer mainly linked by arylglycerol ether bonds between monomeric phenolic units, most of which are not readily hydrolysable.[24] Oxidative enzymes are potential tools for lignin oxidative modification. HRP has been previously used in the preparation of synthetic lignins (DHP) since HRP catalyses the oxidation of monolignols to the corresponding phenoxy radicals.[25] To our knowledge the oxidation pathway of lignin by HRP has never been studied in detail. We report here the design and the development of novel immobilised HRP. More specifically the enzyme was chemically immobilised on alumina particles and then coated by polyelectrolytes bilayers, based on the LbL technique, and used for the oxidative functionalisation of lignin. The aim of our work was to study the reaction pathway of HRP and immobilised HRP on lignins from different origins. The chemical modifications on lignin after the treatments with the immobilised HRP were determined by means of ^{31}P NMR spectroscopy and gel permeation chromatography (GPC).

2. Results and discussion

2.1. Preparation of immobilised HRP

For the immobilisation of HRP we applied a chemical method and then we choose to coat the immobilised catalyst with polyelectrolytes layers using the LbL adsorption technique. Thus HRP from *Armoracia rusticana* was chemically immobilised onto alumina pellets, a material that was choosen for its well known mechanical resistance at high pHs and temperatures.[26] The supports were previously functionalised with glutaraldehyde that covalently links to the enzymes via available amino functions.[27,28] Enzyme cross-linking was avoided by washing several times the supports before adding the enzyme to remove all excess glutaraldehyde. The effectiveness of the immobilisation method was investigated in terms of immobilisation yield, obtained by the analysis of the residual enzymatic activity in the waste waters after the reaction with the supports previously functionalised with glutaraldehyde. An immobilisation yield of 95% with respect to the starting enzyme was obtained, highlighting the high efficiency of the process. Then the alumina-HRP particles were coated by a sequential deposition of alternatively charged polyelectrolytes, in particular the procedure started by the deposition of polystyrene sulphonate (PSS) with a negative charge, followed by the deposition of polyallylamine hydrochloride (PAH) with a positive charge, until the formation of three layers. The deposition started by a negative charge because at neutral pH the alumina-HRP particles were positive charged (Fig. 1). Under these experimental conditions, the immobilised enzyme retained about 68% of its activity with respect to native laccase, as determined by the activity assay, measured spectrophotometrically using ABTS as substrate.[29] This result is comparable with previously obtained results for HRP immobilised on supports activated with glutaraldehyde.[30,31]

Figure 2 shows the scanning electro microscopy of the immobilized and the coated immobilized HRP, A and B, respectively. The coated HRP shows pores on its surface that allow substrate approach to the enzyme active site.

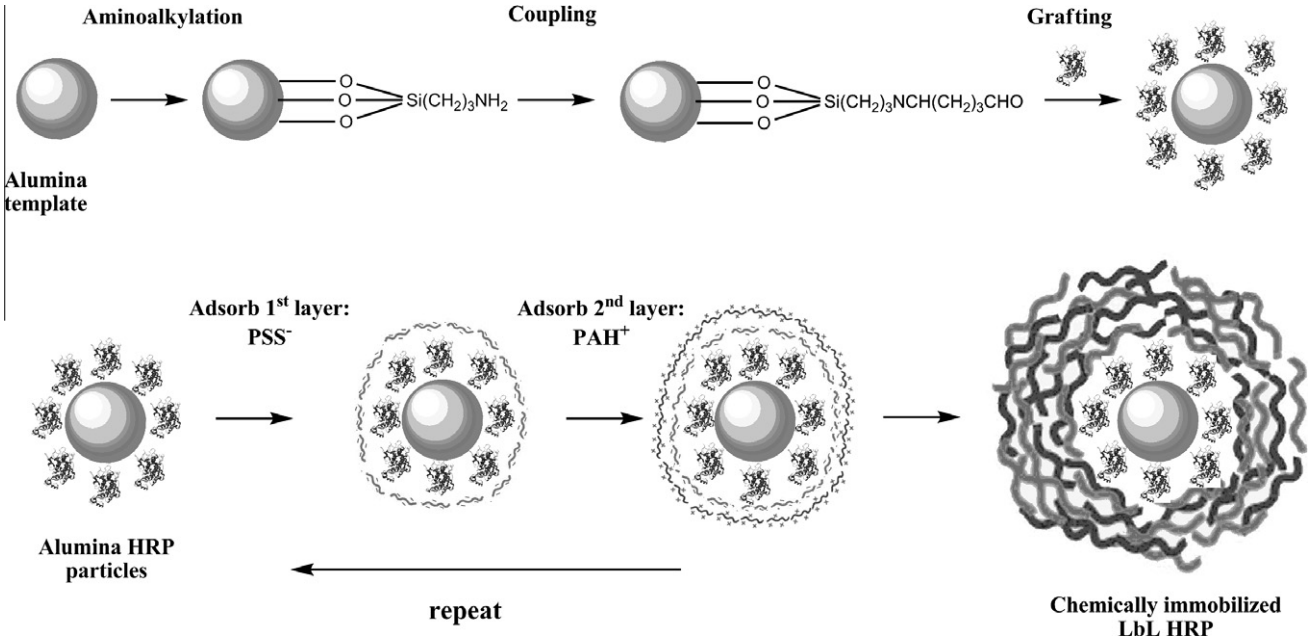

Figure 1. Scheme of preparation of PSS/PAH/PSS-coated HRP/alumina particles: (a) support silanisation; (b) coupling with glutaraldehyde; (c) enzyme cross-linking with the support; (d) layer-by-layer coating of supported HRP.

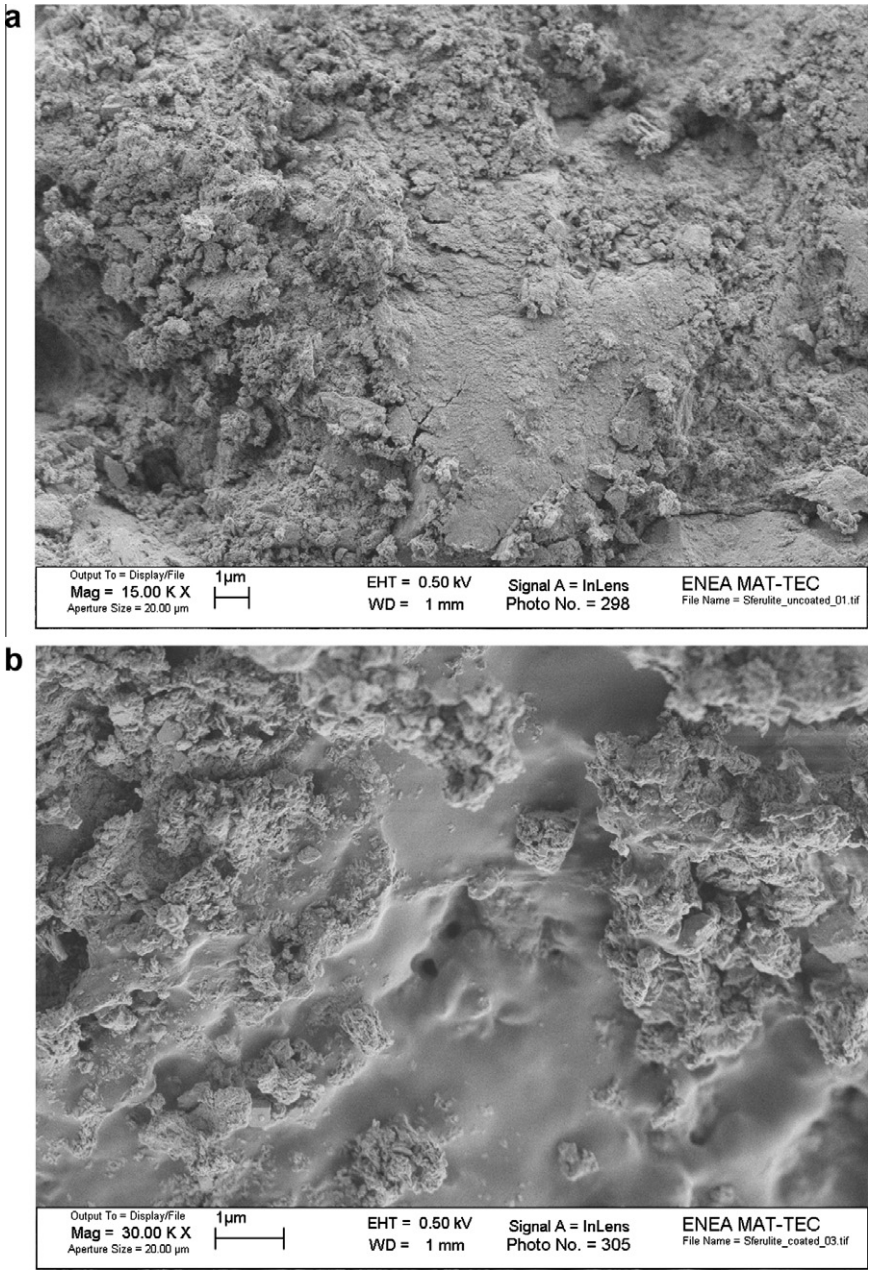

Figure 2. SEM images of HRP/alumina particles before (A) and after (B) polyelectrolyte coating.

2.2. Catalyst recycle

In order to test the possibility of reuse the catalyst, both the free and the immobilised enzymes were allowed to react with ABTS for several successive 12 h batch reactions. In Figure 3 the percentage of retained activity versus catalytic cycle is shown. All the HRPs retained their activity after 10 cycles, although in different proportions. The free enzyme retained 20% of its activity, while the immobilised one retained about 82% of its activity. The percentage of retained activity was calculated with respect to the initial activity for each case. Therefore, it is evident that, in the case of the immobilised enzyme, the polyelectrolytes layers preserved the HRP activity.

2.3. Lignin oxidation with native and immobilised HRP

HRP and the immobilised HRP were used as biocatalyst for the oxidation of two different lignin samples, a black spruce MWL and a residual lignin isolated from softwood kraft pulp. Hydrogen peroxide was used as primary oxidant. MWL is considered the lignin that is more similar to the native one, it is extracted using a mixture of dioxane and water and this procedure is supposed to cause only minor changes in the polymer structure.[32] The M_w of these lignin preparations range between of 15,000–20,000.[33] We choose to use a softwood lignin because of its major resistance to the enzymatic treatments. In fact it has been suggested that guaiacyl lignins, typical of softwoods, restricts fibre swelling and thus accessibility more than the syringyl lignin in the hardwood due to its higher degree of branching.[34] The analysis of this lignin allowed to us to clarify the extention of the oxidative functionalisation versus condensation processes. With respect to the native one, RKL is a highly modified lignin that is produced by chemical pulping processes. It shows a low molecular weight, has a higher phenolic content and a lower methoxyl content and has undergone extensive side-chain oxidation.[35]

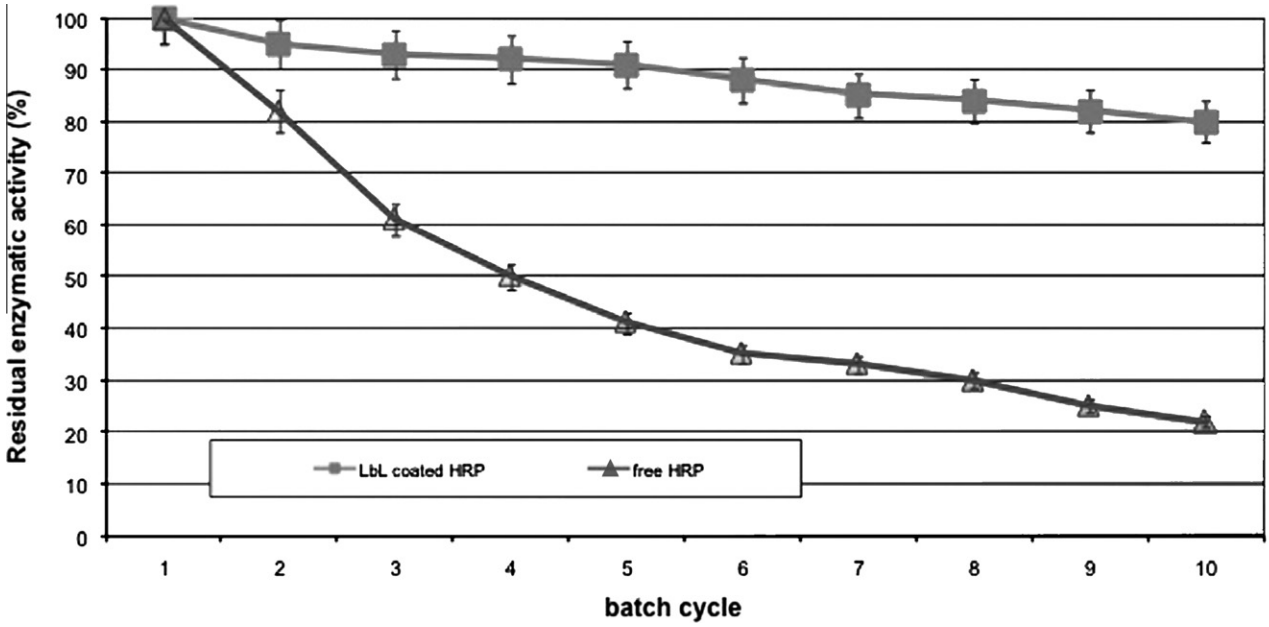

Figure 3. HRP free and coated LbL-HRP residual activity (%) as a function of the number of catalytic cycles. The enzymes were allowed to react with ABTS after 10 successive 12 h batch reactions.

After the HRP treatments on MWL and RKL (80 mg) with immobilised and free enzyme (60 U) were performed, the conversion yield of lignin (g of solubilised lignin/100 g of starting lignin) was calculated (Table 1). In fact, the oxidation increased with the conversion yield due to the changes in the chemical properties of lignin, that became more hydrophilic and soluble in water.[36] Under the same experimental conditions the immobilised enzyme was found more efficient in the oxidation than the free enzyme both on MWL (Table 1, entry 2 vs entry 1) and on RKL (Table 1, entry 4 vs entry 3). More specifically it was possible to recover only about 10% of the starting material using the immobilised HRP in both the lignins, where using the native HRP the recoveries were up to 40%. From these results it is evident that the active site of HRP did not show kinetic barriers to approach the substrate, performing a more extensive oxidative action on lignin with respect to the native enzyme. It can be hyphothesized that the immobilisation process confers a stabilisation effect to the enzyme, improving the oxidative performances.

2.4. ^{31}P NMR characterisation of MWL and RKL after treatment with native and immobilised HRP

In order to extensively clarify the reaction pathway after the HRP treatments both with the native and with the immobilised enzyme, we used an advanced heteronuclear magnetic resonance analysis, the ^{31}P NMR. This technique is an useful tool in that allows both the quantitative and the qualitative characterisation of all labile OH groups present in lignins, namely the aliphatic OH groups, the different phenolic OH and carboxylic acids after the sample in situ phosphytylation. Therefore, by means of this analysis, it was possible to evaluate all the chemical modifications occurred in the lignin structure during the enzymatic treatments. In particular, the samples of residual lignins were phosphitylated with 2-chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane in pyridine/deuterated chloroform mixture (1.6:1.0, v/v ratio) with a suitable internal standard and then subjected to the ^{31}P NMR analysis.[37–39] In Table 2 the quantitative data, for the different functional groups, obtained for MWL and RKL before and after enzymatic treatments, are reported. Both MWL and RKL were deeply modified upon HRP and coated HRP particles treatments. Figure 4 shows possible lignin oxidation pathways. The COOH groups were found increased both by use of HRP and coated HRP particles (Table 2). This indicates an efficient oxidation process. The aliphatic OH groups were not significantly modified, meaning that the side chains were not extensively oxidized (Fig. 4 route A). HRP and supported HRP behave differently from other oxidative enzymes as for example laccases. While laccases show a significant side-chain oxidation process,[40] the HRP reactivity is selective toward the oxidation and cleavage of the aromatic ring as shown in Figure 4 route B. Lignins treated with HRP and coated HRP particles showed increase of condensed OH groups due to oxidative coupling reactions (Fig. 4 route C) on phenolic end groups. As a general trend, the increase in guaiacyl OH groups observed after all the HRP treatments, suggests an increase of hydrophilicity of the polymer and the occurrence of alkyl–phenyl ether bond cleavage reactions which finally result in lignin depolymerisation (Fig. 4 route D). Overall the oxidized insoluble lignins were characterized by a higher content in hydrophilic groups, the treatment with coated HRP particles being more efficient both on MWL and RKL.

Table 1
Conversion of MWL and RKL after treatment with free and immobilised HRP

Entry	Lignin[a,b]	Biocatalyst	Yield[c] (%)
1	MWL	HRP	53.8
2	MWL	Immobilised HRP	89.8
3	RKL	HRP	53.1
4	RKL	Immobilised HRP	88.1

[a] MWL = black spruce milled wood lignin; RKL = softwood residual kraft lignin.
[b] Lignins were oxidised with free or immobilised HRP (60 U) in acetate buffer (40 ml) at 40 °C for 12 h.
[c] Conversion is defined as gram of residual lignin/100 g of starting lignin.

2.5. GPC analysis of MWL after treatment with native HRP and coated HRP particles

The lignins isolated after treatments with the soluble and coated HRP particles were submitted to acetobromination according to a previously reported procedure and successively analysed

Table 2
^{31}P NMR analysis of MWL and RKL before and after the treatments with free and immobilised HRP[a]

Entry	Lignin[b,c]	Biocatalyst	Aliphatic OH	Condensed OH	Guaiacyl OH	COOH
1	MWL	None	6.82	0.21	0.92	0.08
2	MWL	HRP	3.32	0.94	1.47	0.50
3	MWL	Immobilised HRP	6.72	0.68	1.13	0.21
4	RKL	None	2.4	1.38	1.48	0.45
5	RKL	HRP	2.45	1.40	1.53	0.52
6	RKL	Immobilised HRP	4.94	6.36	2.68	3.62

[a] Amount (mmol/g) of aliphatic, phenolic and carboxylic OH groups present on MWL and RKL before and after the enzymatic oxidation, as obtained by quantitative ^{31}P NMR analysis. The ^{31}P NMR data are averages of three phosphitylation experiments followed by quantitative ^{31}P NMR analysis. The maximum standard deviation of the reported data was 2×10^{-2} mmol/g, while the maximum standard error was 1×10^{-2} mmol/g.
[b] MWL = black spruce milled wood lignin; RKL = softwood residual kraft lignin.
[c] Lignins were oxidised with free or immobilised HRP (60 U) in acetate buffer (40 ml) at 40 °C for 12 h.

by gel permeation chromatography in order to investigate the effect of the oxidative treatments on lignin molecular weight distribution. GPC was carried out using a system of columns connected in series calibrated against monodisperse polystyrene standards, monomeric and dimeric lignin model compounds. More specifically 4-(1-hydroxyethyl)-2-methoxyphenol and (3-methoxy-4-ethoxy-2-phenyl)-2-oxoacetaldehyde were used as monomeric and dimeric lignin standard, respectively. Figure 5 shows the GPC analyses before and after the enzymatic treatments. It was evident that, as expected by previous studies on monolignols polymerisations, the M_n and M_W values of the treated lignins were increased by the different HRP treatments. More specifically, the treatment with the coated HRP particles resulted in a more pronounced effect on lignin molecular weight increase with respect to the soluble HRP (Table 3). The GPC analysis of the soluble fractions obtained from lignin oxidation reactions (material not shown), showed only the presence of low molecular weight fragments, while the polymeric portion was entirely localized in the insoluble isolated lignin.

3. Conclusion

In this study we developed a new immobilised HRP, supported onto alumina particles and coated using the LbL absorption technique. The immobilised enzyme showed an increased stability with respect to the native one.

Unexpectedly, the oxidation of both MWL and RKL by HRP showed about 50% lignin conversion, meaning that about 50% of the starting polymer was solubilised and reduced in low molecular weight fragments (i.e., dimers, trimers mainly). The remaining lignin showed a higher molecular weight distribution meaning that a recondensation/ repolymerisation process occurred upon HRP treatment. The oxidized lignins showed a higher content in phenolic OH groups and carboxylic acids. When coated HRP particles were used for lignin oxidative functionalisation the lignin conversion was about 90%. Thus coated HRP particles are more reactive toward lignin depolymerisation with respect to the native enzyme. The higher reactivity might be due to the protection effect of coating polyelectrolytes toward enzyme deactivation. Moreover the immobilised enzyme does not show kinetic barriers to the approach of the polymeric substrate, this is probably due to the irregularity of the polyelectolytes surface, in which pores can be formed. The insoluble lignin recovered after the treatments showed in this case a higher content in aliphatic, phenolic OH groups and carboxylic acids. Overall the process of lignin oxidation by the HRP/ hydrogen peroxide system seems to proceed through both depolymerisation and recondensation of the lignin molecules. Thus we could hypothesize that the depolymerisation process proceeds together with the oxidative coupling slowly depleting the insoluble lignin and increasing the molecular weight of the residual polymer. The constant content of aliphatic OH groups in all treated lignins seems to indicate the occurrence of preferential condensation reactions on the aromatic rings. This is also supported by the increase of carboxylic acids content that could be due to the occurrence of aromatic ring cleavage processes.

On the basis of these data, HRP and coated HRP particles constitute new selective specific biocatalysts for the oxidative functionalisation and depolymerisation of lignin.

4. Experimental

4.1. Reagents

All solvents and chemicals were of analytical grade and high purity. Peroxidase from *A. rusticana* G. (type VI), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), H_20_2 solution (35% w/v), poly(allylamine hydrochloride) (PAH, M_w = 70,000), poly(sodium 4-styrenesulphonate) (PSS, M_w = 70,000), alumina (Al_2O_3) spherical pellets (3 mm diameter), γ-aminopropyltriethoxysilane (γ-APTS), glutaraldehyde and 2-chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane were purchased from Sigma–Aldrich. The polyelectrolyte solutions as well as the buffer solutions were prepared using Millipore MilliQ deionised water (r = 18 MV cm).

4.2. Isolation of residual kraft lignin (RKL) and milled wood lignin (MWL)

MWL was isolated from black spruce. RKL was obtained by using a slightly modified acidolysis procedure as previously reported.[41,42] The yield was 38%, and purity was confirmed by UV and Klason lignin content standard measurements as reported in the literature.[43] MWL was prepared from ultraground extractive-free powder according to Bjorkman's procedure with some modifications.[44]

4.3. Enzyme immobilisation

The enzyme was treated with activated alumina pellets bearing glutaraldehyde linkers previously introduced by the reaction of crude particles with γ-APTS and glutaraldehyde.[45,46] Then, at the end of the reaction, HRP was immobilised on the support.[47] The immobilisation process was carried out according to the following steps. Alumina pellets were silanised with 2% (v/v) γ-APTS in acetone at 45 °C during 20 h. The silanised supports were washed once with acetone and silanised again during 24 h. They were then washed several times with deionised water and dried through air. In the coupling stage the alumina pellets were treated with 2% (v/v) aqueous glutaraldehyde (50%, v/v) during 2 h at room temperature, washed again with deionised water and dried through air. During the grafting, 150 g of support was put in contact with

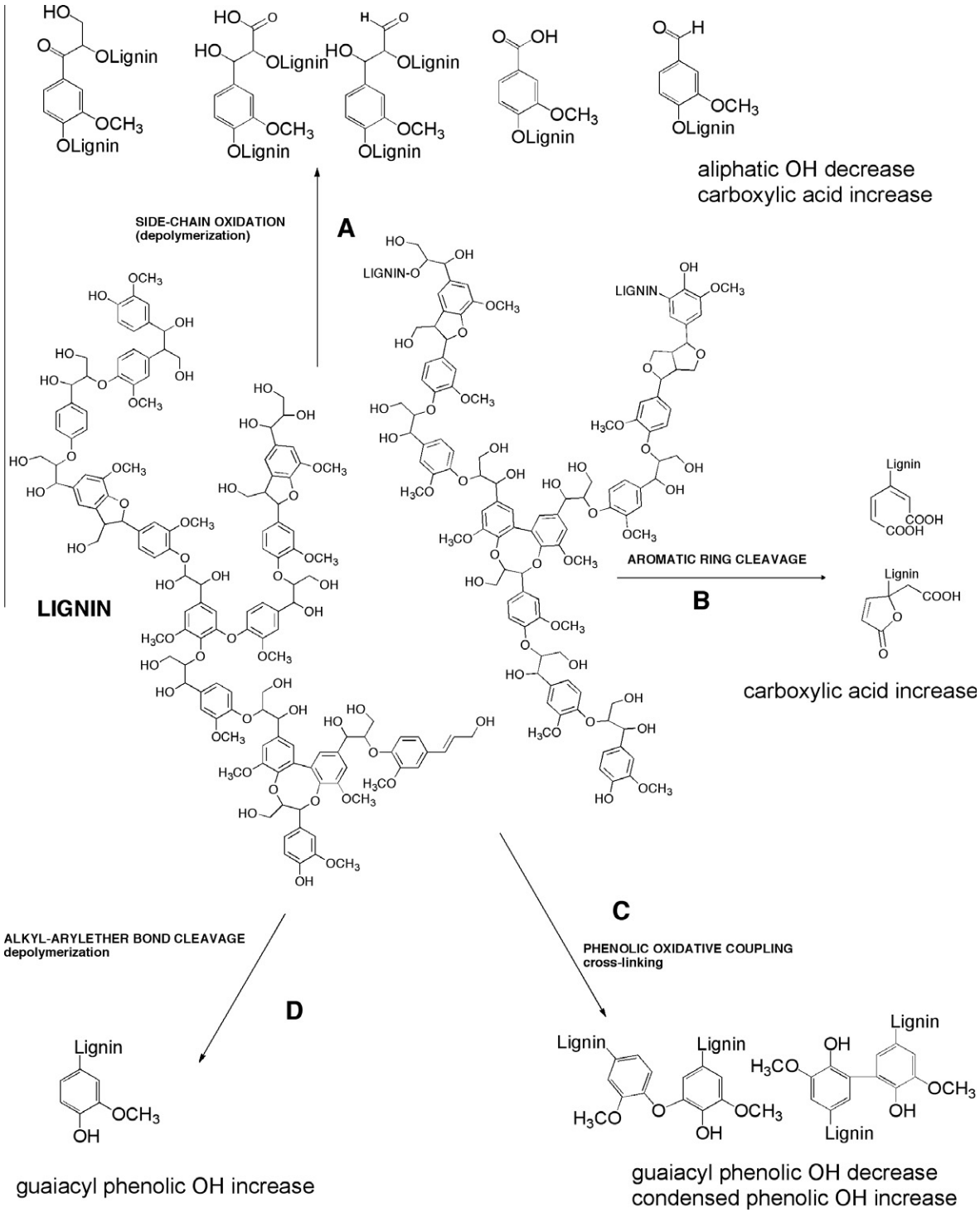

Figure 4. Lignin oxidation pathways.

the enzyme, by immersion, for 48 h at room temperature, in 250 mL of HRP (5000 U/L) solution obtained by dissolving the enzyme in 100 mM citrate buffer pH 7 with 100 mM NaCl. The particles were then washed several times with 0.05 M phosphate buffer (pH 7) until no enzymatic activity was found in the washing solution. In the final stage, LbL coating, the pellets were first washed three times with 0.1 M NaCl and then the sequential deposition of polyelectrolyte (PSS^-, PAH^+) layers onto the alumina particles was performed. Polyelectrolyte solutions (0.01 M) in 0.5 M NaCl were prepared and the supports were immersed inside each solution during 20 min. Since the alumina pellets with immobilised enzyme were positively charged, microcapsules consisting of three layers were created, starting with the negatively charged polyelectrolyte (PSS^-, PAH^+, PSS^-). After each layer, the excess of polyelectrolyte was removed by washing with 0.1 M NaCl. The particles of immobilised LbL HRP were obtained by simple filtration from the reaction mixture.

4.4. Enzyme activity assay

Free HRP activity was determined spectrophotometrically using ABTS as the substrate. The assay mixture contained 0.02 M ABTS,

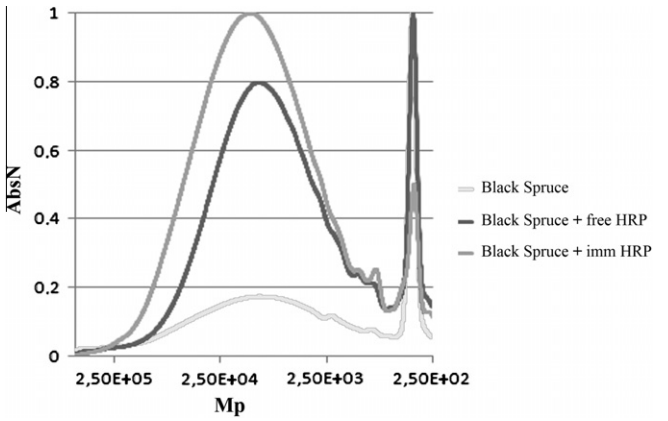

Figure 5. Gel permeation chromatography of black spruce lignin before and after the treatment with free and immobilized HRP. All lignins were previously acetobrominated.

Table 3
Weight-average (M_W) and number average (M_n) molecular weights and polydispersity (M_W/M_n) of lignin samples before and after the enzymatic treatments

Sample	M_W	M_n	M_W/M_n
Black spruce	190,500	26,200	7.27
Black spruce + free HRP	92,500	17,100	5.41
Black spruce + imm. HRP	81,400	22,700	3.59

0.01 M H_2O_2, 0.1 M phosphate buffer pH 7 and an amount of enzyme; the substrate oxidation was followed by an absorbance increase at 405 nm for 2 min for the free enzyme and for 6 min for the immobilized enzyme, as previously reported.[28] One activity unit was defined as the amount of enzyme that oxidised 1 mmol ABTS/min. The immobilisation yield was calculated as the difference between the activity present in the immobilisation solution and that remaining in the supernatant at the end of the adsorption procedure.

4.5. Enzymatic treatments of lignin

Lignin (80 mg) was suspended in acetate buffer pH 6 (40 mL) with 0.5 mM H_2O_2 and treated at 40 °C with the enzyme (60 U) under vigorous stirring to optimise the contact of the solution with air. After 12 h, the mixture was cooled, acidified at pH 3 and centrifuged. The residue was washed with water three times to eliminate solubilised lignin oligomers and then freeze-dried. The residual lignin structure after oxidation was analysed by ^{31}P NMR.

4.6. Quantitative ^{31}P NMR

Derivatisation of the sample with 2-chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane was performed as previously described.[24,37,38] Samples of lignin (30 mg), accurately weighed, were dissolved in a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1.0 (v/v) ratio (0.4 mL). Tetramethylphospholane (0.1 mL) was then added, followed by the internal standard and the relaxation reagent solution (0.1 mL). The NMR spectra were recorded on a Bruker 300 NMR spectrometer using previously published methods.[37,38] To improve resolution, a total of 256 scans was acquired. The ^{31}P NMR data reported in this regard are averages of three phosphitylation experiments followed by quantitative ^{31}P NMR acquisition. The maximum standard deviation of the reported data was 2×10^{-2} mmol/g, while the maximum standard error was 1×10^{-2} mmol/g.[37,38]

4.7. GPC analysis of lignins

Acetobromination of lignin samples for GPC analysis was carried out following the procedure described previously.[47] Briefly, 10 mg of lignin is suspended in acetic acid glacial/acetyl bromide mixture (2.5 ml of 92:8 v/v) and stirred at room temperature. After 2 h the solvent is evaporated under reduced pressure and then the residue is dissolved in 5 ml THF. The GPC analyses were performed using a Shimadzu LC 20AT liquid chromatograph with a SPD M20A ultraviolet diode array (UV) detector set at 280 nm. The sample (20 μl) is injected into a system of columns connected in series (Varian PL gel MIXED-D 5 μm, 1–40 K and PL gel MIXED-D 5 μm, MW 500–20 K) and the analysis is carried out using THF as eluent at a flow rate of 0.50 ml min^{-1}. The GPC system has been calibrated against polystyrene standards (molecular weight range of 890–1.86×10^6 g mol^{-1}) and lignin monomers and model dimers. In particular apocynol and (3-methoxy-4-ethoxy-2-phenyl)-2-oxo-acetaldehyde were synthesized according to literature procedure[48,49] and used as monomer and dimer lignin standard, respectively.

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