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Dye Degradation by Layer-by-Layer Immobilised Peroxidase/Redox Mediator Systems

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Horseradish peroxidase (HRP) was immobilised on Eupergit C 250 L resin coated with poly-electrolytes, or by entrapment inside pre-formed layer-by-layer (LbL) micro-capsules of poly-electrolytes. In these systems, namely HRP/E-LbL, HRPm/LbL and HRPm/LbLp, the native enzyme retained its catalytic activity. Immobilised HRP showed a significant activity in the oxidation of selected azo, quinoline and fluorone dyes with H_2O_2 as the primary oxidant under mild experimental conditions, and HRPm/LbL was the best catalyst. A comparison between the catalytic efficiency of different redox mediators for HRP activity was made by using 1-hydroxybenzotriazole (HOBt), violuric acid (VLA) and veratrylic alcohol (VA). As a general trend, azo dyes were degraded in higher yields, and HOBt was the best mediator for the oxidation. The degradation yield increased on increasing the reaction time and reached the highest value after 12 h, which is comparable with that observed for native HRP. Notably, HRPm/LbL retained its catalytic activity for more runs.

Introduction

Azo, anthraquinone, sulfur,[1a,b] indigoid, trityl(triphenylmethyl) and phthalocyanine derivatives are synthetic dyes extensively used in many fields of industry.[1c] These compounds show significant toxicity,[2] mainly because of the formation of biologically active metabolites, as in the case of carcinogenic amines produced from direct blue 14.[3] Public concern and legislation require the design of environmentally friendly processes to decolourise wastewater after the release of significant amounts of dye into the environment.[4] Different technologies have been developed for this purpose;[5–8] among them, enzymatic processes based on peroxidases have been successfully employed for the degradation of azo dyes, which are the majority of dyes used in industry.[9] In these cases, tests showed a reduction in toxicity after the enzymatic treatment, which highlights the viability of peroxidase in the bio-degradation of textile dyes.[10] Peroxidases are hydrogen peroxide (H_2O_2)-dependent enzymes that catalyse the oxidation of different organic compounds, which include toxic aromatic derivatives and pollulants.[11,12] These enzymes contain the iron protoporphyrin IX as a prosthetic group penta-coordinated in the ferric state to an histidine residue. After reaction with H_2O_2, peroxidases successively form the activated compound I then compound II. An excess of H_2O_2 results in the formation of the inactive compound III, which leads to rapid and irreversible inactivation of the enzyme.[13] Procedures for the immobilisation of peroxidases have been suggested[14] with the aim to increase stability, recyclability and catalytic activity and to overcome the limitations associated with the use of free enzymes in the treatment of complex effluents.[14g] Many valuable strategies for enzyme immobilisation on anchoring supports have been described.[15a,b] These have exploited different approaches such as the introduction of properly bound mediators[15c,f] or by the direct interaction of bound bio-molecules,[15g,h] which depend on the different types of pre-fixed targets. The layer-by-layer (LbL) approach has been applied for the preparation of encapsulated peroxidases.[15i] The LbL technique, first introduced by Decher,[16] is based on the consecutive deposition of alternatively charged poly-electrolytes to form a stable film[17] that protect proteins from denaturing agents without modification of the permeability towards substrates.[18] The loading of the peroxidase within hollow LbL micro-capsules can be favoured by the presence of polyacrylic acid as an additional immobilising phase that has the opposite charge to that of the enzyme at the operating pH.[19] This procedure is useful to stabilise immobilised horseradish peroxidise (HRP) in the oxidation of simple phenol derivatives.[20a,b] The LbL technique can be also applied to the coating of heterogeneous catalysts based on the chemical immobilisation of enzymes on epoxy resins, such as Eupergit C 250 L.[20c] In this paper, we describe the use of heterogeneous catalysts based on the immobilisation of HRP by the LbL technique in the oxidative degradation of different families of dyes. In particular, catalysts in which HRP is immobilised on Eupergit C 250 L resin and coated with layers of elec-

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trolytes and systems in which the enzyme is encapsulated within capsules of poly-electrolytes, or partially occupied by polyacrylic acid, have been used for the degradation of azo, quinoline and fluorone dyes with high efficiency under mild experimental conditions.

Results and discussion

Preparation of LbL-coated Eupergit C 250 L HRP (HRP/E-LbL)

The immobilisation of HRP was initially performed on epoxy-activated acrylic beads of Eupergit C 250 L by using a modification of procedures reported previously for other enzymes.[21,22] Briefly, HRP solution (11 mL, 1634 U, 0.33 mg mL^{-1} in 0.1 M phosphate buffer, pH 6) was treated with dry Eupergit C 250 L (200 mg) at room temperature or lower (5 °C) for 24 or 48 h, respectively (Figure 1, step A). The residual oxyranyl groups of the resin were removed by treatment with a solution of ethanolamine (1.0 M) for 16 h (Figure 1, step B). The beads were collected by filtration and washed with H_2O until no activity was detected in the washings. With the aim to further increase the stability of the supported HRP, the LbL technique was applied with the sequential deposition of poly(sodium 4-styrenesulfonate) (PSS, M_w = 70 000) and poly(allylamine hydrochloride) (PAH, M_w = 70 000). The beads were suspended in negatively charged PSS (2 mg mL^{-1} in 0.5 M NaCl) and treated with positively charged PAH (2 mg mL^{-1} in 0.5 M NaCl). The procedure was repeated for the formation of three layers (Figure 1, steps C–E).

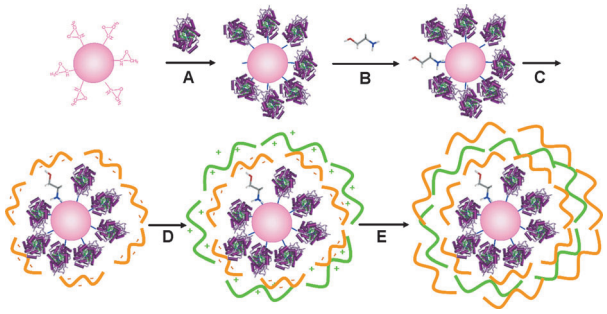

Figure 1. Sequential steps for the preparation of the HRP/E-LbL catalyst.

The effectiveness of the immobilisation procedure was investigated in terms of immobilisation and activity yields. The immobilisation yield is calculated by using Equation (1), in which U_a is the total activity of the enzyme added to the solution and U_r is the activity of the residual enzyme recovered in the washings. The activity yield is calculated by using Equation (2), in which U_x is the activity of the immobilised enzyme. The enzymatic activity was determined by using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as the substrate,[23] and the results are reported in Table 1.

Table 1. Immobilisation and activity yields for HRP/E-LbL.

	Catalyst	Loading[a]	*t* [h]	T [°C]	Immobilisation yield [%]	Activity yield [%]
1	HRP/E-LbL	4412	24	5	43	54
2	HRP/E-LbL	3186	48	5	23	39
3	HRP/E-LbL	4820	24	20	47	59
4	HRP/E-LbL	3513	48	20	32	43

[a] Loading defined as units of enzyme per gram of resin.

$$\text{Immobilisation yield } [\%] = [(U_a - U_r) \times U_a^{-1}] \times 100 \quad (1)$$

$$\text{Activity yield } [\%] = [U_x \times (U_a - U_r)^{-1}] \times 100 \quad (2)$$

The optimal conditions for the immobilisation of HRP were 24 h of treatment at room temperature (Table 1, entry 3). Irrespective of temperature, both the immobilisation and activity yields after treatment for 48 h were lower than those after 24 h, which is probably because of partial mechanical degradation of the beads.

Preparation of micro-encapsulated LbL HRP (HRPm/LbL and HRPm/LbLp)

Hollow poly-electrolyte micro-capsules were produced by the stepwise assembly of PSS and PAH polymeric multi-layers on pre-formed $MnCO_3$ particles.[24,25] The $MnCO_3$ core was then dissolved by treatment with dilute HCl (0.1 M aqueous solution) (Figure 2, steps A–B). The loading of HRP was performed by using a modification of the procedure reported by Gao and co-workers.[15] The hollow capsules were mixed with HRP in acetate buffer (0.1 M, pH 4) at room temperature with different enzyme concentrations and exposure times: 0.5 mL HRP 2 mg mL^{-1} for 24 h, 0.5 mL HRP 4 mg mL^{-1} for 24 h and 0.5 mL HRP 4 mg mL^{-1} for 48 h.[26] At pH 4, some channels are opened in the PSS/PHA capsule, which favours the diffusion of the enzyme from the bulk solution into the core of the capsules (Figure 2, step C). After incubation, the pH of the mixture was

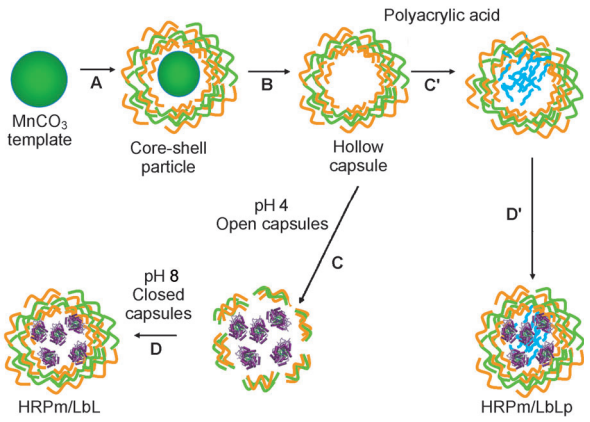

Figure 2. Sequential steps for the preparation of the HRPm/LbL and HRPm/LbLp catalysts.

adjusted to 8.5 with NaOH to close the capsules (Figure 2, step D). Micro-encapsulated LbL-peroxidase (HRPm/LbL) was then recovered by centrifugation of the reaction mixture.

We used the assisted loading technique reported by McShane and Nayak,[27] in which polyacrylic acid acts as an anionic internal immobilising phase (Figure 2, step C′).

The loading procedure was repeated with enzyme concentrations and exposure times similar to that described above to yield the micro-encapsulated LbL-peroxidase/polyacrylic acid system (HRPm/LbLp) (Figure 2, step D′). In this case, the internal anionic polyacrylic acid matrix is responsible for the electrostatic-driven uptake of cationic HRP (operating pH 5, $pI_{HRP} = 8.8$).[27] The immobilisation and activity yields of HRPm/LbL and HRPm/LbLp are reported in Table 2. The highest immobilisation yield was obtained without polyacrylic acid by using HRP (0.5 mL, 4 mg mL^{-1}) for 24 h (Table 2, entry 1). Prolonged reaction times did not afford better results.

Table 2. Immobilisation and activity yields for HRPm/LbL and HRPm/LbLp.

	Catalyst	*t* [h]	*T* [°C]	Immobilisation yield [%]	Activity yield [%]
1	HRPm/LbL	24	20	56	54
2	HRPm/LbL	48	20	53	50
3	HRPm/LbLp	24	20	42	41
4	HRPm/LbLp	48	20	39	37

This trend, observed for both types of catalysts, can be explained by the existence of a limit to the loading value within the capsules. The similarity between the immobilisation and activity yields indicates that most of the immobilised enzyme maintains its catalytic activity after the treatment. Irrespective of the loading conditions, the activity yield was higher for HRPm/LbL than HRPm/LbLp. The amount of HRP entrapped per capsule in both systems was evaluated by cell counting by using a Bürker chamber. The empty capsules loaded 8.7×10^{-9} mg of HRP per capsule versus 6.5×10^{-9} mg in the presence of the matrix (Table 3). These data suggest that the polyacrylic acid matrix inside the capsule is possibly sterically encumbered.

Table 3. Amount of HRP per capsule with and without the polyacrylic acid matrix.

	Catalyst	*t* [h]	Encapsulated HRP [%]	[mg]	No. capsules	HRP per capsule [mg]
1	HRPm/LbL	24	56	0.79	9×10^7	8.7×10^{-9}
2	HRPm/LbL	48	53	0.75	9×10^7	8.3×10^{-9}
3	HRPm/LbLp	24	42	0.59	9×10^7	6.5×10^{-9}
4	HRPm/LbLp	48	39	0.55	9×10^7	6.0×0^{-9}

Oxidation of dyes with HRP/E-LbL, HRPm/LbL and HRPm/LbLp catalysts

The HRP/E-LbL, HRPm/LbL and HRPm/LbLp catalysts were used in the oxidation of a large panel of dyes, which included azo dyes, amaranth (**1**), tartrazine (**2**), cochineal red A (**3**), carmoisine (**4**) and sunset yellow (**5**), a quinoline dye, quinoline yellow (**6**) and a fluorone dye, erythrosine (**7**) (Figure 3).

Initially, **4** was selected as a probe for the degradation process under both homogeneous and heterogeneous conditions. The reactions were performed by taking the experimental conditions described by Ferreira-Leitão and co-workers as a reference.[28] A solution of **4** (33 μM, 2,4 mL 0.1 M phosphate buffer, pH 8) was treated with native or immobilised HRP (1.3 μM) and H_2O_2 (165 μM, **4**/H_2O_2 = 1:5) at 37 °C for 1 h with orbital shaking (150 rpm).[29] Control experiments were performed without HRP or by using empty capsules and un-loaded Eupergit C250L resin: no dye oxidation was observed in both cases. The reactions were followed by spectro-photometry at the maximum wavelength for **4** ($\lambda_{max} = 515$ nm). In accordance with procedures reported in the literature,[30] the degradation yield was calculated by using Equation (3), in which Abs_{t_0} is the absorbance at detection time t_0 and Abs_{t_1} is the absorbance at detection time t_1 (in this case $t_1 = 60$ min), and the results are shown in Figure 4.

$$\text{Degradation yield } [\%] = [(Abs_{t_0} - Abs_{t_1})/Abs_{t_0}] \times 100 \quad (3)$$

The immobilised HRP systems showed a lower reactivity than the native enzyme (the maximum degradation yield for native HRP was 85% after 10 min) probably because of the presence of a kinetic barrier for the diffusion of the substrate. Among the heterogeneous systems, micro-encapsulated HRPs were more reactive than the Eupergit-supported enzyme, and HRPm/LbL was the best catalyst (56% degradation yield after

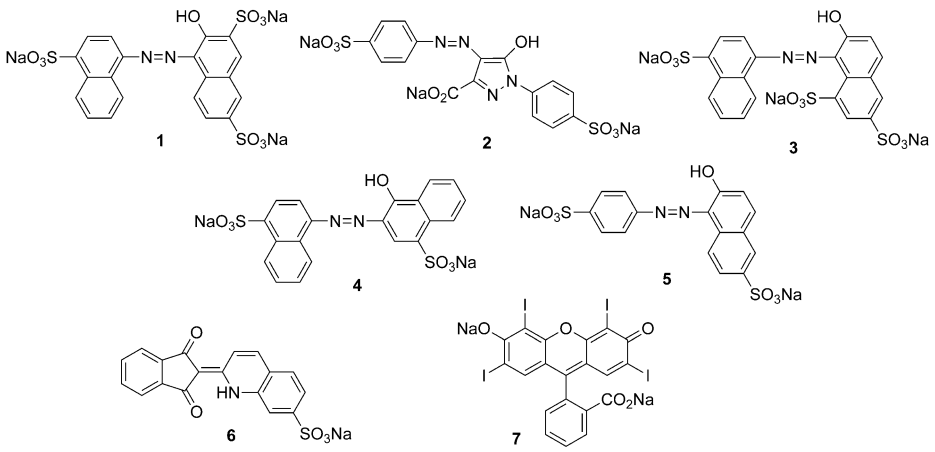

Figure 3. Structures of selected azo, quinoline and fluorone dyes.

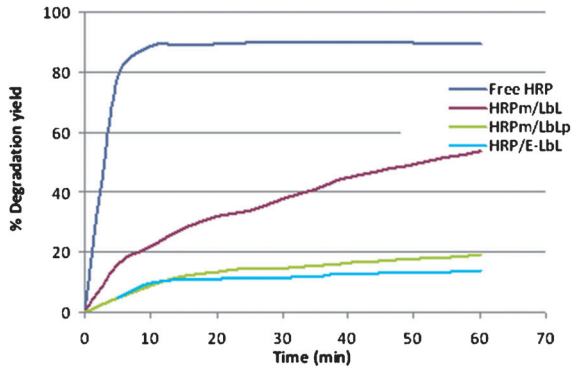

Figure 4. Degradation yield of **4** with free and immobilised HRP.

60 min). Better results were not obtained by increasing the concentration of H_2O_2 (up to 330 μM H_2O_2; **4**/H_2O_2 = 1:10). Notably, HRP/E-LbL was partially coloured at the end of the reaction. In this case, the reactive component is the resin Eupergit C 250 L, and the LbL layer is un-reactive (as shown by the absence of staining for HRPm/LbL and HRPm/LbLp). In accordance with data reported previously,[31] there is the possibility of residual un-reacted oxirane groups on Eupergit C 250 L, which would allow it to interact with dye molecules. The treatment of HRP/E-LbL with ethanolamine during the preparation of the catalyst was probably not sufficient to eliminate these secondary reactions. Further attempts to de-activate the resin with other reagents (diethylamine and 2-mercaptoethanol) did not lead to better results.

The LbL capsule wall has semi-permeable properties:[32] it is permeable for small molecules, such as dyes and ions, whereas high-molecular-weight molecules are excluded under most conditions. However, ionic strength can, in principle, modify the interaction between poly-electrolytes and favour swelling processes.[33] With the aim to evaluate the possible role of the ionic strength on the efficiency of the reaction, the oxidation of **4** with HRPm/LbL (for 60 min) was repeated in the presence of 0.1 M and 0.25 M NaCl solutions. Under these experimental conditions, degradation yields of 58 and 60% were obtained, respectively (a degradation yield of 57% was detected without NaCl). From these results, we concluded that under conditions with a higher value of ionic strength, the de-colourisation of **4** was not significantly affected.

To evaluate the effect of immobilisation on the activity of HRP, the kinetics of the degradation of **4** were studied in the presence of HRPm/LbL and native HRP. The initial rates were calculated from Lineweaver–Burk plots (1/V vs. 1/[S]). The Michaelis–Menten constant (K_m) and the maximum reaction rate (V_{max}) were determined from the intercepts of the *x* and *y* axes, respectively. HRPm/LbL had a K_m value (0.490 mM) higher than HRP (0.278 mM), which suggests a reduced affinity of HRPm/LbL for the substrate. This pattern was confirmed by the V_{max} value, which was lower for HRP/LbL than for HRP (187 vs. 241 U mg^{-1}). Similar trends of K_m values have been reported for HRP immobilised on different carriers, which were attributed to possible mass-transfer limitations.[34]

The oxidation of dyes **1**–**3** and **5**–**7** was successively performed with the most reactive HRPm/LbL catalyst under optimised experimental conditions. The results, which include data for the oxidation of **4** and native enzyme as references, are reported in Figure 5 (for a reaction time of 60 min).

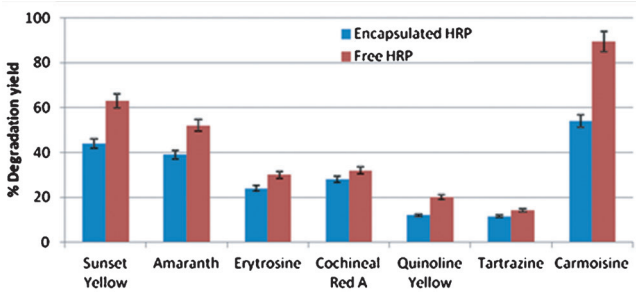

Figure 5. Degradation yield of **1**–**7** with free and immobilised (HRPm/LbL) HRP.

In general, HRPm/LbL always showed a reactivity lower than the native enzyme probably because of the kinetic concerns cited above. However, a relevant substrate specificity was observed. In particular, the azo dyes (except **2**) and **7** were the most degraded compounds (degradation yields in the range 32–89% for the native enzyme and 28–54% for HRPm/LbL), and **6** was only slightly oxidised. Interestingly, HRPm/LbL retained its reactivity for longer reaction times as shown in the oxidation of **1**, **4**, **5** and **7**, which were the most reactive substrates, for different durations (1, 3, 20 and 36 h, Table 4). The highest activity was reached within the first 3 h, after which the system achieved a steady state with degradation yields generally comparable with that observed for the native enzyme (for clarity, Table 4 reports only the degradation yield for the native enzyme after 36 h). Generally, the difference between the oxidation efficiency of HRPm/LbL and that of the native enzyme is significant for short reaction times (60 min), whereas it tends to decrease until it reaches comparable values for longer reaction times. This trend is probably because of the influence of substrate diffusion on the kinetics of the heterogeneous system. Moreover, the ability of the heterogeneous system to maintain its reactivity for longer reaction times confirms the beneficial effect of the LbL coating.

Table 4. Degradation yields of dyes **1**, **4**, **5** and **7** at different reaction times with HRPm/LbL.

Dye	Degradation yield [%]				
	t = 1 h	t = 3 h	t = 20 h	t = 36 h	t = 36 h[a]
1	39	49	56	58	62
5	44	54	59	61	89
4	54	74	77	80	92
7	24	29	31	34	38

[a] Reaction performed with native HRP.

Degradation of dyes with HRPm/LbL in the presence of redox mediators

Once the efficiency of HRPm/LbL in the oxidation of dyes had been evaluated, we started to study the effect of redox mediators on the catalytic activity of the encapsulated enzyme. Mediators are low-molecular-weight compounds that act as electrons shuttles, which favour the oxidation of recalcitrant substrates. The mediators diffuse far away from the active site and, by mechanisms different from those of the native enzyme, enable the oxidation of the target compounds. Two major factors determine the efficiency of mediators: the redox potential and the specific interaction with the active site.[35] Violuric acid (VLA, redox potential (E^0) = 0.97 V vs. standard hydrogen electrode (SHE))[36] and phenothiazine-10-propionic acid (PPA, E^0 = 0.71 V vs. SHE) are excellent mediators in the de-colourisation processes with lignin peroxidase.[37–39] Here we tested three mediators with different E^0 values: veratrylic alcohol (VA, E^0 = 1.36 V vs. SHE), used previously for the activation of HRP in the polymerisation of cardanol,[40] 1-hydroxybenzotriazole (HOBt, E^0 = 1.13 V vs. SHE) and VLA. The catalytic cycle for HRP in the presence of redox mediators requires that the enzyme reactive intermediates compound I (oxyferryl π-cation radical heme) and compound II (oxyferryl heme), formed after the reaction with H_2O_2, are regenerated through the production of the oxidised counterparts of the mediators.[41] In general, a mediator must have a redox potential sufficiently low to allow its oxidation by the enzyme but, at the same time, it should be high enough to promote the oxidation of substrates.[35] HOBt, VA and VLA show a similar mechanism for oxidation based on a radical H-abstraction (HAT) pathway.[42] The initial step of the HAT process is the conversion of the mediator into a radical cation that quickly de-protonates to give the corresponding aminoxyl radical. This intermediate subsequently removes a H atom from the substrate and finally evolves towards the oxidation product.[43–45]

In the HAT process, the redox potential of the substrate has a negligible effect on the reactivity. Initially, dyes **1–7** (33 μM, 2.4 mL, 0.1 M phosphate buffer, pH 8) were oxidised under homogeneous conditions with native HRP (1.3 and 165 μM H_2O_2) with mediators (33 μM) at 37 °C for 60 min to optimise the experimental conditions. The degradation yield of the dyes is generally increased in the presence of mediators, and the best results were obtained in the presence of HOBt (Table 5). Among the three mediators, VA was the least efficient and VLA generally showed an intermediate activity similar, in some cases, to that of HOBt (see for example Table 5, entries 17–20). This reaction pattern suggests that mediators with a value of E^0 between 0.93 and 1.13 V are more effective in the H_2O_2 oxidation of dyes catalysed by native HRP. In accordance with data reported previously, the de-colourisation of dyes in the presence of mediators occurred by the degradation of the aromatic ring and by cleavage of the azo group,[46,47] as confirmed by the presence of traces of aromatic amine and sulfanilic acid derivatives in the reaction mixture.[10] The de-colourisation varies with the nature of the dye, and the highest degradation yields were observed in the oxidation of **2** (Table 5, entries 5–8), **3** (entries 9–12) and **5** (entries 17–20). This effect was less noticeable in the oxidation of **1** and **4**, probably because the degradation yield was already high in the absence of mediators (Table 5, entries 1–4 and 13–16, respectively). Notably, a slight but significant increase in degradation with HOBt for both the recalcitrant **6** and **7** was observed (Table 5, entries 21–24 and 25–28, respectively).

Table 5. Oxidation of dyes **1–7** with native HRP in the presence of redox mediators HOBt, VLA and VA.

	Dye	Mediator[a]	Degradation yield [%][b]
1	**1**	none	85
2	**1**	HOBt	90
3	**1**	VLA	86
4	**1**	VA	80
5	**2**	none	17
6	**2**	HOBt	41
7	**2**	VLA	26
8	**2**	VA	24
9	**3**	none	24
10	**3**	HOBt	87
11	**3**	VLA	50
12	**3**	VA	23
13	**4**	none	85
14	**4**	HOBt	93
15	**4**	VLA	92
16	**4**	VA	90
17	**5**	none	62
18	**5**	HOBt	96
19	**5**	VLA	96
20	**5**	VA	90
21	**6**	none	20
22	**6**	HOBt	31
23	**6**	VLA	27
24	**6**	VA	18
25	**7**	none	24
26	**7**	HOBt	58
27	**7**	VLA	27
28	**7**	VA	26

[a] Reactions performed with native HRP (1.3 μM and 165 μM H_2O_2) and mediators (33 μM) at 37 °C for 60 min. [b] Degradation yield calculated by using Equation (3).

The oxidation of **1–7** was successively performed with the most reactive HRPm/LbL and HOBt mediator system under optimised experimental conditions by using dye (33 μM), immobilised HRP (1.3 μM), HOBt (33 μM) and H_2O_2 (165 μM) at 37 °C for 1 h, and the results, which include data for HRPm/LbL without a mediator for reference, are reported in Table 6. As expected, the heterogeneous catalyst was less efficient than native HRP (Table 5 vs. Table 6). Irrespective of the dye used, the reactivity of HRPm/LbL increased in the presence of HOBt and reached significant values (i.e., a degradation yield greater than 50%) in the case of **1**, **4** and **5** (Table 6, entries 2, 8 and 10). A slight increase in degradation with HOBt was also observed with dyes more recalcitrant to oxidation, which include **2**, **3**, **6** and **7** (Table 6, entries 4, 6, 12 and 14). Moreover, the efficiency of degradation with HRPm/LbL and HOBt increased on increasing the reaction time, which is reported in Table 6 for **5** for 6 and 12 h (Table 6, entries 15–16). Under these experimental conditions, a degradation yield comparable with the native

Table 6. Oxidation of dyes **1**–**7** with HRPm/LbL with and without HOBt.

	Dye	Catalyst	Degradation yield [%][b]
1	**1**	HRPm/LbL	39
2	**1**	HRPm/LbL/HOBt[a]	56
3	**2**	HRPm/LbL	16
4	**2**	HRPm/LbL/HOBt[a]	25
5	**3**	HRPm/LbL	24
6	**3**	HRPm/LbL/HOBt[a]	45
7	**4**	HRPm/LbL	54
8	**4**	HRPm/LbL/HOBt[a]	62
9	**5**	HRPm/LbL	44
10	**5**	HRPm/LbL/HOBt[a]	56
11	**6**	HRPm/LbL	16
12	**6**	HRPm/LbL/HOBt[a]	21
13	**7**	HRPm/LbL	24
14	**7**	HRPm/LbL/HOBt[a]	34
15	**5**	HRPm/LbL/HOBt[c]	72
16	**5**	HRPm/LbL/HOBt[d]	82
17	**5**	HRPm/LbL/HOBt[e]	80
18	**5**	HRPm/LbL/HOBt[e]	81
19	**5**	HRPm/LbL/HOBt[e]	79
20	**5**	HRPm/LbL/HOBt[e]	76

[a] Reactions performed with HRPm/LbL (1.3 μM and 165 μM H_2O_2) and mediators (33 μM) at 37 °C for 60 min. [b] Degradation yield calculated by using Equation (3). [c] Reaction performed for 6 h. [d] Reaction performed for 12 h. [e] Reusability of HRP/mLbL for more runs.

HRP was observed (Table 5, entry 18 vs. Table 6, entry 16). To evaluate the reusability of HRPm/LbL, we repeated the oxidation of **5** under the same experimental conditions. After 12 h, HRPm/LbL was recovered, washed and reused with fresh substrate and HOBt. HRPm/LbL retained its high activity with a degradation yield of more than 70% after five runs (Table 6, entries 16–20).

Finally, even if the treatments with bio-catalysts are efficient for de-colourisation, it is necessary to evaluate the occurrence of the formation of toxic or recalcitrant products during the degradation process. To evaluate this point, we used the standard plating technique with *Pseudomonas putida*.[48] As a selected case, the reaction mixture obtained by treatment of **4** with HRPm/LbL and HRPm/LbL/HOBt (after removal of the catalyst and deactivation of excess H_2O_2) was used as a single carbon source for the inoculum grown from *Pseudomonas putida* on plates with nutrient agar (2%). The plates were incubated at 30 °C for 5 d. After incubation, the growth was continuously monitored for up to 7 d. In both the HRPm/LbL- and HRPm/LbL/HOBt-treated plates, the formation of colonies was observed after the second day of incubation and, subsequently, profuse growth of colonies was seen. Similar results were obtained for the control plates (treatment with native HRP). This shows that a specific toxicity or resistance to bio-degradation does not appear after treatment with heterogeneous systems.

Conclusions

HRP/E-LbL, HRPm/LbL and HRPm/LbLp were synthesised by immobilisation of HRP on Eupergit C 250 L further coated with poly-electrolytes or by entrapment inside pre-formed LbL micro-capsules of poly-electrolytes. In all systems, HRP retained its catalytic activity. These systems showed a significant activity in the oxidation of selected azo, quinoline and fluorone dyes with H_2O_2 as the primary oxidant under mild experimental conditions, and HRPm/LbL was the best catalyst. In the case of HRP/E-LbL, the catalyst was partially coloured at the end of the reaction, which suggests the presence of side processes that are impossible to avoid by using the preventive procedure for the de-activation of the epoxy resin. A comparison of the catalytic efficiency of different redox mediators for HRP activity was made by using HOBt, VLA and VA as co-catalysts for dye oxidation. In general, azo dyes were degraded in the highest yield, and HOBt was the best mediator for the oxidation. The degradation yield increased on increasing the reaction time and reached the highest value after 12 h, which is comparable with that observed for native HRP. We observed that the de-colourisation efficiency was related to the different chemical structures of the dyes. Although it is difficult to suggest the actual molecular mechanism that operates in the heterogeneous mixture, some hypotheses can be made based on data reported in the literature. For example, in the case of azo dyes, enzymatic degradation without the assistance of a redox mediator occurred only with free hydroxyl-substituted derivatives, whereas non-phenolic dyes, except for trifluoromethyl-substituted ones, are degraded only in the presence of redox mediators.[49] These data can explain the low reactivity observed for HRPm/LbL with **6** and **2** (the hydroxyl groups of which are salified at the pH value used in the experiments). From the data reported in Table 6, it can be concluded that the dyes that have a hydroxyl group in the *ortho* position relative to the azo bond (**1**, **3**–**5**) were efficiently degraded by treatment with HRPm/LbL in conjunction with HOBt. As the system is complex (that is, the double interaction of enzyme–mediator vs. mediator–substrate), it is possible to suggest only a simplified correlation between the oxidation efficacy and the redox potential of the dyes[50] based on the fact that all mediators tested were active (their redox potentials are low enough to allow their oxidation by HRP) and that they showed a similar HAT mechanism. For example, in the oxidation of **4**, the highest reactivity was obtained with the VLA ($E^0 = 0.97$ V) and HOBt ($E^0 = 1.13$ V) systems, which are characterised by a redox potential closer to that of **4** ($E^0 = 0.42$ V, at pH 8[51]) than that of VA ($E^0 = 1.36$ V). Finally, HRPm/LbL retained its catalytic activity for more runs, which suggests the real possibility of its employment in the treatment of coloured wastewater under environmentally friendly conditions.

Experimental Section

Materials: Horseradish peroxidase (HRP, EC 1.11.1.7) lyophilised powder, 1-hydroxybenzotriazole hydrate (HOBt), ammonium bicarbonate (NH_4HCO_3), ethanol, Eupergit C 250 L, sodium 2-mercaptoethanesulfonate, thioacetic acid and *N*,*N*-diethylamine were purchased from Fluka. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide water solution (H_2O_2, 35 wt%), manganese(II) sulfate monohydrate ($MnSO_4{\cdot}H_2O$), poly(sodium 4-styrenesulfonate) (PSS, $M_W = 70\,000$), poly(allylamine hydrochloride) (PAH, $M_W = 56\,000$), acrylic acid, ammonium persulfate ($(NH_4)_2S_2O_8$), disodium ethylenediaminetetraacetate (EDTA,

sodium salt), dialysis membrane and dyes **1**–**7** were purchased from Sigma–Aldrich. All spectro-photometric measurements were performed by using a Varian Cary50 UV/Vis spectro-photometer equipped with a single-cell peltier thermo-static cell holder. Spectro-photometric data were analysed by using Cary WinUV software. The water used in all experiments was Milli-Q water with a resistivity higher than 18.2 MΩ cm.

HRP activity assay: The enzymatic activity of free and immobilised HRP was determined by using ABTS as a substrate.[23] The assay mixture contained ABTS (1.6 mM), phosphate buffer (0.1 M, pH 6 or 8), H_2O_2 (0.8 mM) and a suitable amount of enzyme (40 U, corresponding to approximately 10 mg of resin in the case of the immobilised enzyme; see Table 1 for the loading factor). In the case of immobilised enzyme, the catalyst was removed prior to the analysis to avoid any interference in the determination of absorbance. The oxidation of ABTS was followed by an absorbance increase at 405 nm. The enzyme activity is expressed in units [U], which is equivalent to the number of μmol of ABTS oxidised per minute at 25 °C under the specified conditions. The amount of immobilised enzymatic units was calculated by the difference between the units loaded and those recovered in the washings. The activity of immobilised HRP was calculated from the activity of the native enzyme measured under the same conditions.

Determination of protein concentration: The protein concentration was determined spectro-photometrically at 595 nm by using Bradford reagent and bovine serum albumin (BSA) as the standard.[52,53] The amount of HRP immobilised was calculated by the difference between the units loaded and that recovered in the washings.

Dye assays: The optimum wavelength of each dye was determined by scanning a solution of the dye (33 μM) over a range of 200–800 nm: **1** λ_{max} = 521 nm, **2** λ_{max} = 425 nm, **3** λ_{max} = 506 nm, **4** λ_{max} = 515 nm, **5** λ_{max} = 480 nm, **6** λ_{max} = 412 nm, **7** λ_{max} = 525 nm.

Preparation of $MnCO_3$ particles: Ethanol (5 mL, final concentration 5% v/v) was added with vigorous stirring into a mixture of equal volumes of NH_4HCO_3 (50 mL, 0.16 M) and $MnSO_4$ (50 mL, 0.0016 M). The system was incubated at 50 °C for 1 h to yield $MnCO_3$ microparticles with an average diameter of 5.5 μm.[24,54] At the end of the reaction, the precipitated $MnCO_3$ particles were collected by centrifugation and washed three times with deionised water. The particles were air-dried at 60 °C for 24 h and stored in a micro-centrifuge tube.[55]

Preparation of PSS/PAH micro-capsules: The stepwise assembly of the PSS/PAH multi-layer on the $MnCO_3$ micro-particles was conducted by using a protocol described elsewhere.[26,56,57] Briefly, PSS and PAH solutions (20 mL, 2 mg mL^{-1} in 0.5 M NaCl) were alternately added to $MnCO_3$ particles (40 mg) suspended in H_2O (1 mL). Each poly-electrolyte layer was adsorbed for 20 min, and the particles were collected by centrifugation and washed with H_2O three times before the addition of the next layer to remove any poly-electrolytes that remained in the supernatant solution. After the deposition of eight alternate poly-electrolyte layers, the $MnCO_3$ cores were dissolved by three treatments with HCl (0.1 M). The capsules obtained were washed with H_2O until the capsule suspension was maintained at pH 6 and then treated with EDTA solution (0.01 M) to remove residual Mn ions. The hollow micro-capsules obtained were rinsed with H_2O three times to remove excess EDTA.

HRP encapsulation in the presence of polyacrylic acid: The polyacrylic acid (PAA) matrix was formed within the capsule cavities as described elsewhere.[27] Briefly, the polymerisation mixture was prepared with acrylic acid monomers (2 mL, 99%) with $(NH_4)_2S_2O_8$ (2 mL, 2 mg mL^{-1}) as the initiator. The hollow capsules were suspended in H_2O (2 mL), added to the polymerisation mixture (1:1 v/v) and incubated at 60 °C for 1 h until the change in viscosity of the mixture was observable. After polymerisation, the capsules were washed twice with H_2O and dialysed overnight against H_2O to remove both un-reacted monomer and $(NH_4)_2S_2O_8$ from the capsule suspension. The capsules were mixed with HRP in acetate buffer (0.1 M, pH 4) at RT as follows: HRP (0.5 mL, 2 mg mL^{-1}) for 24 h for the first encapsulation, HRP (0.5 mL, 4 mg mL^{-1}) for 24 h for the second encapsulation and HRP (0.5 mL, 4 mg mL^{-1}) for 48 h for the third encapsulation. After incubation, the pH of the mixture was adjusted to 8.5 by using NaOH and, after 1 h, the suspension was centrifuged and washed with phosphate buffer (0.1 M, pH 8) until no enzymatic activity was found in the supernatant. The amount of immobilised HRP was calculated as previously described.

HRP encapsulation without polyacrylic acid: The previously synthesised hollow capsules were mixed with HRP solution (2 mL, 2 mg mL^{-1}, 0.1 M acetate buffer) at RT as follows: HRP (0.5 mL, 2 mg mL^{-1}) for 24 h for the first encapsulation, HRP (0.5 mL, 4 mg mL^{-1}) for 24 h for the second encapsulation and HRP (0.5 mL, 4 mg mL^{-1}) for 48 h for the third encapsulation.[26] After incubation, the pH of the mixture was adjusted to 8.5 by using NaOH and, after 1 h, the suspension was centrifuged and washed with phosphate buffer (0.1 M, pH 8) until no enzymatic activity was detected in the washings. The amount of immobilised HRP was calculated as previously described.

HRP immobilisation on Eupergit C250l: An HRP solution (0.33 mg mL^{-1} in 0.1 M phosphate buffer, pH 6) was treated with dry Eupergit C250L (200 mg) at RT or lower (5 °C) and gently shaken for 24 or 48 h, respectively.[21,22] At the end of the coupling period, the beads were collected by filtration, washed with H_2O until no activity was detected in the washings and finally suspended in binding buffer. Before use, the beads were freeze-dried to remove the buffer. The units of HRP coupled on the resin were calculated by the difference between the amount loaded and that recovered in the washings calculated from Bradford and ABTS assays. The residual groups of the Eupergit C250L were removed by treatment with a solution of ethanolamine. Briefly, loaded beads (66 mg) were added to ethanolamine (500 μL, 1 M) and incubated for 16 h. After the incubation time, the beads were washed with H_2O and equilibrated with 0.1 M phosphate buffer (pH 6).

Dye oxidation: General procedure: reaction mixtures (2.4 mL) in phosphate buffer (0.1 M, pH 8), which contained dye (33 μM), free or immobilised HRP (1.3 μM) and H_2O_2 (330 μM), were incubated at 37 °C for different reaction times.[28] Control experiments without HRP, H_2O_2 or supports (i.e., empty capsules or un-loaded Eupergit C250 L) were also performed. No dye oxidation was observed in all of the control experiments. The reactions were followed by using spectro-photometry at the typical wavelength of each dye.

Kinetic studies: Kinetic experiments were performed in a series of vials (c = 20 mg L^{-1}) with the pH of the aqueous phase, HRP concentration (2.94 units) and H_2O_2 dose (0.2 μM) kept constant. The reaction mixtures were agitated by using a horizontal shaker at 150 rpm, and the residual dye concentration in the aqueous phase was analysed after centrifugation (5000 rpm, 5 min, 24 °C). Each vial was removed at a pre-determined time and the residual dye concentration in the aqueous phase was estimated. The initial velocities were calculated from Lineweaver–Burk plots (1/V vs. 1/[S]).

K_m and V_{max} were determined from the intercepts at the *x* and *y* axes, respectively.

Oxidation of dyes by HRP/redox mediator systems: Reaction mixtures (2.4 mL) in phosphate buffer (0.1 M, pH 8), which contained dye (33 μM), free or encapsulated HRP (1.3 μM), H_2O_2 (330 μM) and a solution of selected redox mediator (VA, HOBt or VLA, 33 μM), were incubated at 37 °C in cuvettes. The reactions were followed by spectro-photometry at the typical wavelength of each dye.

Toxicity with *Pseudomonas putida*: The enhanced degradation of the dye in the aqueous phase after the HRP-catalysed reaction was assessed by adopting a standard plating technique (control and enzyme-treated dye on 2% agar plates). The inoculum was grown on plates (control plate, i.e., treated with native HRP and either HRPm/LbL- or HRPm/LBL/HBOt-treated plates after the removal of the catalyst and degradation of the excess H_2O_2) with nutrient agar (2%) along with a dye as a single carbon source to understand the enhanced degradation. The plates were incubated at 30 °C for 5 d. After incubation, growth was continuously monitored for up to 7 d. On the HRPm/LbL- and HRPm/LBL/HBOt-treated plates, the formation of colonies was observed after the second day of incubation and, subsequently, the profuse growth of colonies was seen. Similarly, on the control plate, growth was observed only after 2 d of incubation.

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Keywords: dyes/pigments · biocatalysis · oxidation · polymerization · layered compound

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