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Immobilized redox enzymatic catalysts: Baeyer–Villiger monooxygenases supported on polyphosphazenes

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ABSTRACT

A novel method has been employed for the selective covalent co-immobilization of a Baeyer–Villiger monooxygenase (phenylacetone monooxygenase from *Thermobifida fusca*) and a NADPH recycling enzyme (glucose-6-phosphate dehydrogenase) on the same polyphosphazene carrier for the first time starting from $\{NP[O_2C_{12}H_{8-x}(NH_2)_x]\}_n$ (x ranging from 0.5 to 2) using glutaraldehyde as connector. In all cases the preparation was active and it was found that the optimum proportion of amino groups in the starting polyphosphazene was 0.5 per monomer. The immobilized biocatalysts showed similar selectivity when compared with the isolated monooxygenase, demonstrating the potential of this novel type of immobilizing material, although their recyclability must still be improved.

1. Introduction

Economy and productivity have been for decades the main criteria to evaluate industrial processes. However, in the last few years, the design of chemical routes which involve less quantity of reagents and generate less waste and hazardous materials is becoming a very important issue, embracing the so called 'Green Chemistry' [1]. In this sense, more and more chemical transformations are changing from a 'stoichiometric mode' to a catalytic one. The use of these methodologies diminishes costs and enhances productivity affording better atom economy, higher energy efficiency, and less waste production [2]. Among all the catalytic protocols developed, biocatalytic reactions have recently gained more relevance due to the mild conditions employed and the high chemo-, regio-, and/or stereoselectivities achieved [3–6]. Although undoubtedly enzymes offer several advantages regarding other catalysts, they still suffer from certain drawbacks such as high price and relatively low stability and medium flexibility. These problems can be minimized by employing techniques of enzyme immobilization [7–9]. When, *e.g.* a biocatalyst is covalently attached to a support in an active form, it can be reused, the enzyme stability can be improved and the final product is enzyme-free. An ideal support should offer various characteristics like high chemical stability and temperature resistance.

In this sense, polyphosphazenes are good model polymers because their physical and chemical properties can be easily tuned by selecting the appropriate functionalities linked to the phosphorous atom [10,11]. The synthesis of functionalized polyphosphazenes by secondary reactions on pendant side groups is a fruitful alternative to the classical macromolecular substitution with nucleophiles carrying the desired functional groups [10–12]. Due to their high chemical resistance, these derivatives posses a great potential for biotechnological applications and, in fact, novel materials derived from this type of polymer have recently been used in the field of medicine [13,14]. Functionalized polyphosphazenes have proven to be an efficient material to covalently attach different types of enzymes like trypsin or glucose-6-phosphate dehydrogenase on a support of $[NP(OPh)_2]_n$ on an alumina carrier [15], or an invertase on spherical particles of $[NP(OCH_2CF_3)_2]_n$ [16]. In another contribution, an urease was encapsulated on a hydrogel derived from poly[bis(methoxyethoxyethoxy)phosphazene] through irradiation with γ-rays to cross-link the polymer and trap the biocatalyst [17]. Recently, we have developed the synthesis of an amino polyphosphazene derivative that

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could be used as tunable support to immobilize different enzymes. The polyphosphazene $\{NP[O_2C_{12}H_{7.5}(NH_2)_{0.5}]\}_n$ was prepared by a nitration–reduction sequence and was successfully employed as carrier to attach *Candida antarctica* lipase B and alcohol dehydrogenase from *Rhodococcus ruber* (ADH-A). Using these supports we could demonstrate that the immobilized lipase was stable in organic solvents while the immobilized alcohol dehydrogenase (ADH) was used in aqueous solution [18]. These biocatalysts could be recycled several times in both aqueous and organic media.

Oxidoreductases are very interesting catalysts to achieve highly stereoselective reactions. One of the members of this enzyme family are the Baeyer–Villiger monooxygenases (BVMOs, EC 1.14.13.x) [19–22], a group of NAD(P)H dependent flavoproteins. Apart from the Baeyer–Villiger reaction, these enzymes are able to catalyze several oxidative processes with high regio- and stereoselectivities employing molecular oxygen as mild oxidant. One of the main drawbacks for scaling-up BVMO-catalyzed reactions is the need of the expensive nicotinamide cofactor NAD(P)H. For this, usually another enzyme like glucose-6-phosphate dehydrogenase (G6PDH) is employed coupled with the BVMO to allow the cost effective utilization of a catalytic amount of the cofactor. Recently, a more sophisticated 'self-sufficient' approach was described by covalently fusing both BVMO and a recycling enzyme [23,24]. In this case, several BVMOs were linked to a phosphite dehydrogenase (PTDH), which catalyzes the oxidation of phosphite into phosphate by which it reduces $NADP^+$ into NADPH.

There are few examples concerning BVMO immobilization. Zambianchi and co-workers co-immobilized cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. and an ADH from *Thermoanaerobium brockii* (ADHTB) on Eupergit C to carry out the sulfoxidation of thioanisole. Under these conditions, the BVMO half-life time increased and several reuses could be performed [25]. In a previous report, Abril et al. showed another CHMO immobilization example, but no information concerning its recycling was given [26]. Very recently, recombinant *Escherichia coli* whole-cells overexpressing cyclopentanone monooxygenase (CPMO) were encapsulated in polyelectrolyte complex capsules which rendered a biocatalyst more active and stable than the corresponding free cells [27]. In this study we explored the potential of several amino polyphosphazenes to covalently attach phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* [28] and G6PDH. PAMO is an attractive BVMO since it is a thermostable protein able to selectively oxidize a range of ketones [29–33] and sulfides [34–37]. We report for the first time the attachment of PAMO on several polyphosphazene carriers $\{NP[O_2C_{12}H_{8-x}(NH_2)_x]\}_n$ (**5**–**7**) and their properties as new biocatalysts. These polymers were used to immobilize to the same chain both PAMO and glucose-6-phosphate dehydrogenase (G6PDH) enzymes.

2. Experimental

2.1. General

Ketone **14**, ester **15**, sulfides **19** and **21**, sulfoxide **20**, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* were purchased from commercial sources. Compounds **17**, **18**, **22**, **23**, and **24** were synthesized as previously shown [33,37]. PAMO from *T. fusca* was purified as previously described [28]. 1 unit (U) of PAMO oxidizes 1.0 μmol of phenylacetone to benzyl acetate per minute at pH 9.0 and 25 °C in the presence of NADPH. Polyphosphazenes **1** [38,39], **2**–**4** [40], and **5** [18] were synthesized as previously described. Tetrahydrofuran (THF) was treated with KOH and distilled twice from Na in the presence of benzophenone.

The infrared (IR) spectra were recorded with a Perkin-Elmer FT Paragon 1000 spectrometer. NMR spectra were recorded on Bruker NAV-400, DPX-300, AV-400, and AV-600 instruments. The ^1H and $^{13}C\{^1H\}$ NMR spectra in deuterated dimethylsulfoxide (DMSO-d^6) are given in δ relative to trimethylsilane (TMS) (DMSO at 2.51 ppm and 40.2 ppm, respectively). $^{31}P\{^1H\}$ NMR are given in δ relative to external 85% aqueous H_3PO_4. The C, H, N, analyses were performed with an Elemental Vario Macro. Tg was measured with a Mettler DSC Toledo 822 differential scanning calorimeter equipped with a TA 1100 computer. Thermal gravimetric analyses (TGA) were performed on a Mettler Toledo TG 50 TA 4000 instrument. The polymer samples were heated at a rate of 10 °C min^{-1} from ambient temperature to 900 °C under constant flow of nitrogen or under air. Gas chromatography (GC) analyses were performed on a Hewlett Packard 6890 Series II chromatograph. High performance liquid chromatography (HPLC) analyses were carried out with an UV detector at 210 nm using a chiral HPLC column. For the determination of enzymatic conversions and stereoselectivities, see Supplementary Data.

2.2. Synthesis of $\{NP[O_2C_{12}H_{8-x}(NH_2)_x]\}_n$ (**5**–**7**)

To the prepared Lalancetteĭs reagent [18,41], THF (50 mL) and a solution of $\{NP[O_2C_{12}H_{8-x}(NO_2)_x]\}_n$ **2**–**4** [40] (2 g, 9.96 mmol) in THF (50 mL) were added and the final volume was increased adding 100 mL of THF. The mixture was then refluxed with stirring for 24 h (the formation of a solid in the walls of the flask was observed). After cooling to room temperature, 10% (v/v) aqueous HCl (5 mL) were added and stirring was continued for 7 h. The resulting mixture was filtered and the solid was stirred with 10% aqueous NaOH (100 mL) for 24 h. The solid was separated by filtration, washed with plenty of water until neutral pH, and dried at 40 °C under vacuum for 3 days (76–87% yield).

6 ($x = 1$): IR (KBr) cm^{-1}: 3430, 3366 m.br (ν NH), 3064 w (ν CH arom.), 1623 br.m, 1499 m, 1479 m (ν CC arom., δ NH), 1384 sh.m (typical of biphenoxyphosphazenes, not assigned), 1346 m, 1267 s, 1246 s, 1192 vs (ν NP), 1096s (ν P-OC), 1040 w, 1013 w (not assigned), 943–923 s.br (δ P-OC), 820 v.w (not assigned), 785 s, 751 s (δ CH arom.), 606 m, 536 m.br (other). ^1H NMR (ppm, DMSO-d^6): 6.6–7.4 v.br (aromatic protons), 4.5 v.br (NH_2). ^{31}P NMR (ppm, DMSO-d^6): −5.0 br. Analysis (Calcd.): C 55.2 (59.0), N 10.9 (11.4), H 3.5 (3.7), sulfur retained 2%. TGA (from ambient to 900 °C): Continuous loss from 400 to 800 °C; final residue 47% (under N_2), 13% (under air). Tg: 132 °C.

7 ($x = 2$): IR (KBr) cm^{-1}: 3435, 3367 m.br (ν NH), 3065 w (ν CH arom.), 1623 br.m, 1521 m, 1499 m (ν CC arom., δ NH), 1389 sh.m (typical of biphenoxyphosphazenes, not assigned), 1347 s, 1231 s, 1194 vs (ν NP), 1120 s, 1094 s (ν P-OC), 1041 w, 1027 w (not assigned), 945–925 s.br (δ P-OC), 834 w, 781 s, 744 s (δ CH arom.), 636 m, 578 m.br (other). Analysis (Calcd.): C 47.8 (55.6), N 14.8 (16.2), H 2.8 (3.9), sulfur retained 2%. TGA (from ambient to 900 °C): Continuous loss from 300 to 800 °C with a significant loss at 365 °C; final residue 37% (under N_2), 19% (under air). Tg: 149 °C.

2.3. Immobilization of PAMO on polyphosphazenes **5**–**7** to obtain **11**–**13**

Polyphosphazene **5**–**7** (60 mg) was added to a saturated solution of $(NH_4)_2SO_4$ (9 mL) and a solution of glutaraldehyde (1 mL, 2.5%, v/v) in phosphate buffer 50 mM pH 7. Then it was mixed under magnetic stirring during 2 h at 50 °C. The solid obtained was filtered off, washed with phosphate buffer 50 mM pH 7 (3 × 1 mL), and dried under vacuum, affording **8**–**10** (71–85% yield).

Intermediate **8**–**10** (20 mg) was added to 1 mL of Tris–HCl buffer 50 mM pH 9 with PAMO (20 μL, 100 μM, 1 U) and was orbitally stirred (250 rpm) at 40 °C during 15 h. Afterwards, the polymer