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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 Lalancetteǐs 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 was filtered, washed with Tris–HCl buffer 50 mM pH 9 (4 × 0.5 mL) and dried under vacuum. These formulations were obtained as fine powders, and no control of the particle size (microns) could be achieved, so the effects of this parameter in the catalytic activity could not be evaluated. In all cases, buffer employed to wash **11** showed remaining enzymatic activity (≤5%), demonstrating that PAMO was in excess when it was immobilized on polyphosphazene **8**.

### 2.4. Co-immobilization of PAMO and G6PDH on polyphosphazene 5 to obtain 16

To a suspension of **8** at 40 °C in 1 mL of Tris–HCl buffer 50 mM pH 9, 20 μL of PAMO (100 μM, 1 U) and 10 μL of G6PDH (10 U) were added. The reaction mixture was stirred under orbital shaking at 40 °C and 250 rpm overnight. Then, it was centrifuged and washed with buffer Tris–HCl 50 mM pH 9 (3 × 1 mL) and dried under vacuo. This formulation was obtained as fine powder, and no control of the particle size (microns) could be achieved, so the effects of this parameter in the catalytic activity could not be evaluated.

### 2.5. Enzymatic oxidation of phenylacetone employing 11–13 as biocatalyst

20 mg of biocatalysts **11**–**13** were placed in an Eppendorf tube and then 500 μL of Tris–HCl buffer 50 mM 1 mM NADPH pH 9, D-glucose-6-phosphate (20 μL, 500 mM), phenylacetone (5 μL, 1 M in DMSO), and G6PDH (5 μL, 10 U) were also added. The reaction was shaken at 30 °C and 250 rpm during 24 h. Afterwards, the reaction was extracted with ethyl acetate (2 × 0.6 mL), and the organic phase was dried over $Na_2SO_4$. Conversion was measured by GC (see Supplementary Data).

### 2.6. Enzymatic oxidation employing 16 as biocatalyst

20 mg of biocatalyst **16** were placed in an Eppendorf tube and then 500 μL of Tris–HCl buffer 50 mM 1 mM NADPH pH 9, D-glucose-6-phosphate (20 μL, 500 mM), and the substrate (5 μL, 1 M in DMSO) were also added. The reaction was shaken at 30 °C and 250 rpm during 24 h. Afterwards, the reaction was extracted with ethyl acetate (2 × 0.6 mL), and the organic phase was dried over $Na_2SO_4$. Conversions and enantioselectivities were measured by GC or HPLC (see Supplementary Data).

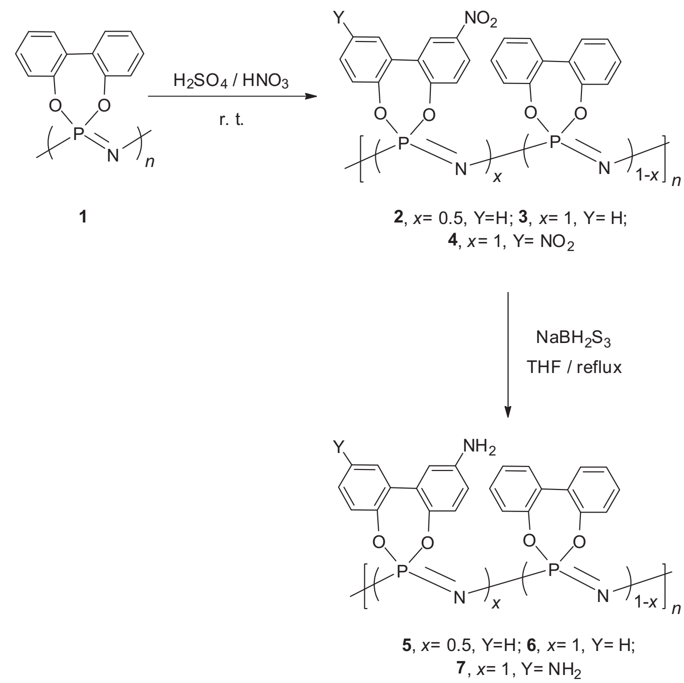
### 2.7. Recycling study for the oxidation of 14 with polyphosphazenes 11 or 16

20 mg of biocatalysts **11** or **16** were placed in an Eppendorf tube and then 500 μL of Tris–HCl buffer 50 mM 1 mM NADPH pH 9, D-glucose-6-phosphate (20 μL), phenylacetone (5 μL, 1 M in DMSO), and G6PDH (5 μL) in case of polyphosphazene **11** were also added. The reaction was shaken at 30 °C and 250 rpm during 24 h. Afterwards, the reaction was extracted with ethyl acetate (2 × 0.6 mL), and the organic phase was dried over $Na_2SO_4$. To avoid material loss, the next cycles were performed in the same eppendorf tube, adding to the polymer the rest of reagents as mentioned before. Finally, conversion was measured by GC (see Supplementary Data).

## 3. Results and discussion

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

The synthesis of polymers **5**–**7** (Scheme 1) was achieved as previously described starting from polyphosphazene **1** [38,39], by selective nitration with a sulfonitric mixture at room temperature during 1.5 h [40], followed by reduction with Lalancette's reagent

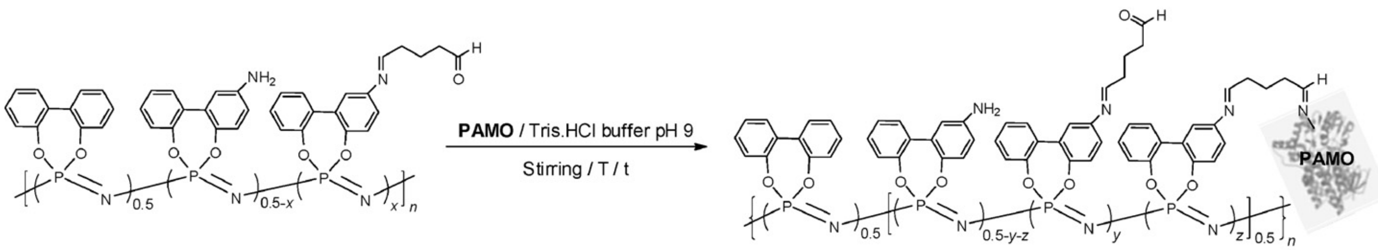



**Scheme 1.** Synthesis of polyphosphazenes **5**–**7**.

[41] under reflux of THF (see Section 2). The composition and structure of the products was confirmed by the analytical and spectroscopic data. The IR showed the expected bands at >3300 $cm^{-1}$ ($\nu$ NH) and the broadness of the band around 1620 $cm^{-1}$ due to the overlapping with the NH-bending absorption. The relative intensities of the IR signals at 1527 and 1478 (in $cm^{-1}$) was consistent with the *x* value in the chemical formula of the nitro polyphosphazenes **2**–**4** [40]. It must be mentioned that experimental carbon contents lower than the calculated values are frequent in polyphosphazenes containing amino groups and is related to high char residues, as confirmed by the TGA data. The presence of the $NH_2$ group was also observed in the $^1H$ NMR (DMSO-$d^6$) as a very broad signal centered between 4.5 and 4.9 ppm. The very low solubility of these compounds in THF prevented the measurement of the $M_w$. In fact, there was clear a trend: the more percentage of amino groups present in the polymer, the more insoluble it was. TGAs from r.t. to 900 °C showed a continuous weight loss from 300 to 800 °C with a significant loss between 360 and 420 °C obtaining as final residues 37–47% under $N_2$ or 13–19% under air.

### 3.2. Immobilization of PAMO on polyphosphazene 5. Study of the carrier structure

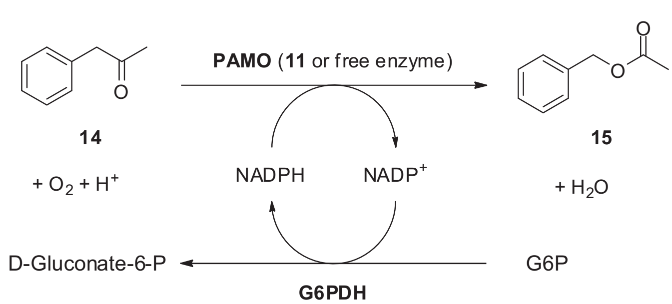
Due to our previous experience with polymer **5** [18], we started the study with this carrier. Thus, it was envisaged to use the same covalent methodology in view of the excellent results obtained for ADH-A and CAL-B. Initially, amino polyphosphazene **5** was activated employing glutaraldehyde due to the fact that this molecule posses two reactive aldehydes moieties, being a logical choice to act as linker between the polymer and the protein. In the same conditions as previously shown [18], polyphosphazene with glutaraldehyde **8** was obtained in high yields. In the following step, we tried to link PAMO (polymer **11**, Scheme 2) in the same conditions that were found for ADH-A. Therefore, a PAMO solution in Tris–HCl 50 mM pH 9 buffer, due to the fact that this is the optimal pH for this enzyme [28], was added to **8** under magnetic stirring at 5 °C for 15 h.




**Scheme 2.** General strategy to covalently link PAMO to activated polyphosphazene **8**.

To find out if a biocatalyst has been attached in a support, several techniques like IR spectroscopy or NMR can be employed, although in our specific case, the very low solubility of these preparations led us to use an indirect probe such as the enzymatic activity. After 15 h of incubation, the immobilized PAMO preparation **11** was tested using as a model reaction the oxidation of phenylacetone **14** into benzyl acetate **15** in Tris–HCl buffer. For this, G6PDH and G6P were added to recycle the cofactor in a 'coupled enzyme' approach (Scheme 3). Although the observed enzymatic conversion (7%), was much lower than the one obtained with purified PAMO (>97%), the result clearly demonstrated the anchoring of the enzyme to the polyphosphazene. In this case, in order to compare the enzymatic activities of both preparations, the quantity equivalent to one enzyme unit (1 U) was used.

To optimize the enzyme immobilization (Scheme 2), different parameters, like reaction time, temperature, and type of shaking were tested when mixing **8** with PAMO. Due to the fact that PAMO is thermostable [42], higher temperatures for its immobilization were tried. Thus, when the reaction was done at room temperature during 15 h, the enzymatic conversion was doubled (14%), although still very low. This process was repeated at 40 °C during 15 h (18% conv.) or 24 h (20% conv.), at 50 °C for 15 h (11% conv.), and 60 °C for 15 h (≤3% conv.), but still enzymatic conversions were not high enough. Since it has been described that in some cases the magnetic stirring can be harmful for enzymes [43], orbital shaking was selected to immobilize PAMO at 40 °C on polyphosphazene with glutaraldehyde **8**. After one night of incubation, we observed comparable activity to that obtained with the purified enzyme (>97%). Higher incubation temperatures did not improve this result. To ensure that unspecific adsorption did not occur, underivatized polymer **5** was incubated with PAMO and then washed with buffer. The resulting treated carrier showed very low enzymatic activity (~10%). Another control experiment was the incubation of polyphosphazene with PAMO **11** in Tris–HCl buffer 50 mM pH 9 for 24 h at 30 °C and 250 rpm. After that time, the polymer was filtered off, washed, and the oxidation of **14** was performed again, obtaining 84% of conversion after 24 h. The small reduction of the activity can be ascribed to some inactivation of the protein during the whole process.




**Scheme 3.** PAMO-catalyzed oxidation of phenylacetone **14** employing a 'coupled enzyme' approach.

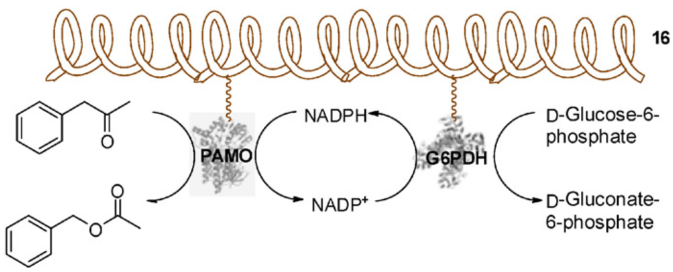
In order to measure the units of PAMO attached per mg of polyphosphazene, a calibration curve plotting U of PAMO vs enzymatic conversion in the oxidation of **14** was made (see Supplementary Data), obtaining that 8 mU $mg^{-1}$ of polyphosphazene were present in this preparation.

It has been recently found that an immobilized lipase on silica presented a stability improvement using 1,4-diamines as a space arm between two molecules of glutaraldehyde [44]. This could be attributed to a lower steric hindrance between the carrier and the protein, therefore minimizing undesirable interactions between other functional groups on the carrier surface. Starting from activated polyphosphazene with glutaraldehyde **8**, 1,4-diaminobutane was firstly inserted [18]. Due to the higher nucleophilicity of the aliphatic diamine, the first and second coupling reactions were done at room temperature, while the last one was achieved at 40 °C in the previous optimal conditions. This new biocatalytic preparation showed very low enzymatic conversion (9% after 1 h) in comparison to **11** (77% after 1 h). Therefore, these conditions were dismissed.

Considering the potential hydrolytic instability of the imine bond present in the biocatalyst **11** that could diminish its activity within time, a mild reductive agent such as sodium borohydride ($NaBH_4$) was employed to transform these bonds into stable amino-type ones [18]. Unfortunately, the material obtained by treating **11** with a solution of $NaBH_4$ (0.026 M) in water for 30 min at 0 °C did not show any measurable enzymatic conversion, indicating the total inactivation of the monooxygenase during the reduction protocol. So, it was not further considered.

Once the best conditions of immobilization for PAMO on **5** were established, the next step was the study of the polyphosphazene carrier. At this point, we were interested in the possible effect of enhancing the quantity of linkers present in the polymer. Thus, polyphosphazenes $\{NP[O_2C_{12}H_{8-x}(NH_2)_x]\}_n$ with a higher (**6**, $x = 1$ and **7**, $x = 2$) proportion of amino groups were synthesized in high yields using the same protocol as for polymer **5** (Scheme 1). The key step to control the amino ratio was the molar quantity of nitric acid (ranging from 0.1 to 0.4 M) employed in the nitration step. Subsequently, the activation with glutaraldehyde and immobilization of PAMO were performed in the best conditions found for **5** to obtain biocatalysts **12** and **13**. In order to compare the effect of the support, the oxidation reaction of phenylacetone **14** was done in the same reaction conditions as for polymer **11**.

After 1 h of reaction, the three supported enzymes gave a similar conversion of 80%, showing that a percentage of $x = 0.5$ was a sufficient quantity to get full reactivity. As an increase in the presence of the amino groups did not lead to an improvement in the enzymatic conversion, polyphosphazene **11** was selected as suitable biocatalyst to perform further experiments. It is important to notice, however, that polymers **6** and **7** can also be selected as carriers to immobilize PAMO and that the presence of a higher




**Scheme 4.** Schematic representation of the co-immobilization of PAMO and G6PDH on a polyphosphazene carrier (**16**).

number of reactive moieties does not interfere with the enzymatic activity.

### 3.3. Co-immobilization of PAMO and G6PDH on polyphosphazene *5*

Until now PAMO was successfully attached on different polyphosphazenes using G6PDH and G6P to recycle internally the cofactor. In case of recycling the supported biocatalyst, it would be highly desirable to co-immobilize the recycling enzyme to diminish costs, thus being only necessary the addition of G6P to perform the biocatalytic reaction in each cycle (polyphosphazene **16**, Scheme 4). This novel biocatalyst would resemble a 'self-sufficient' redox enzyme [23,24], where the BVMO and the dehydrogenase are covalently linked.

Thus, a suspension at different proportions of PAMO and G6PDH was mixed with **8** in Tris–HCl buffer 50 mM pH 9 in the best immobilization conditions to afford **16** (Scheme 4). In order to study the effect of the presence of G6PDH in the immobilization process, the PAMO/G6PDH ratio was varied among 1–10 U, 1–5 U, and 1–2.5 U. As mentioned before, to perform the biooxidation of **14**, only G6P was added into the buffer. It was observed that a lower proportion of the recycling enzyme led to a lower biocatalytic conversion, probably due to the fact that less protein was present in the polyphosphazene, and therefore, the overall process was slower. While a conversion around 80% was achieved with a proportion 1–10 U after 1 h, it diminished when a proportion 1–5 U was employed (58% conv.), and was even lower (40% conv.) in the case of 1–2.5 U. Therefore, for subsequent experiments a PAMO/G6PDH proportion 1–10 U was used to prove that G6PDH was able to work when supported on a polyphosphazene carrier, as previously shown for other similar supports [15]. Attempts to quantify the proportion of PAMO and G6PDH in **16** were achieved. Thus, treatment of the polymer with SDS to extract the flavin of the BVMO into the solution [28], and then measurement of the UV spectra at 450 nm (maximum of flavin absorption) was recorded in order to determine the decrease of the intensity of this signal comparing polyphosphazenes **11** (only PAMO) and **16** (PAMO plus G6PDH). Unfortunately, the quantity of PAMO attached to these polymers was too low and any absorption was observed in any case. Another probe was done making this experiment with the same concentration of pure protein in solution, but any absorption of the flavin was observed, demonstrating again that the quantity of enzyme (and therefore flavin), was under the detection limit.

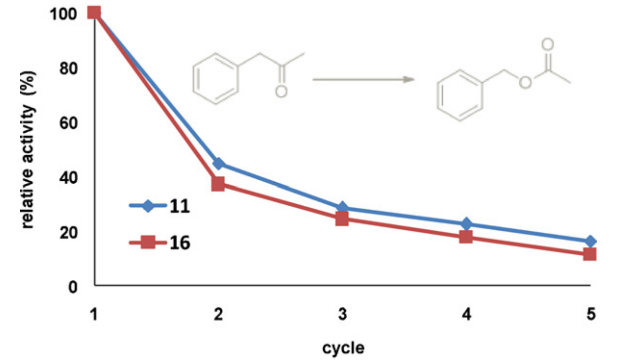
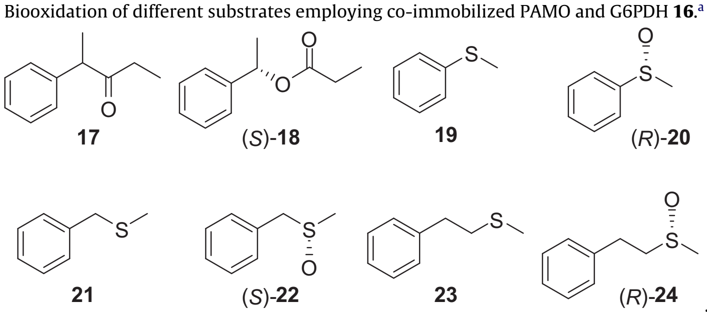



**Fig. 1.** Recycling study of **11** and **16** in the enzymatic oxidation of **14**.

### 3.4. Recycling capacity and stereoselectivity

Enzyme immobilization presents as main advantage the possibility of its recycling during several times, therefore minimizing the cost of the enzymatic process. This is especially interesting in the case of oxidoreductases, since these proteins work mainly in aqueous solutions and are soluble in this medium, avoiding the possibility of their reuse. Herein, we have studied the recycling capability of both **11** and **16** applied to the biooxidation of phenylacetone (Fig. 1). It was observed that both preparations were active

**Table 1**
Biooxidation of different substrates employing co-immobilized PAMO and G6PDH **16**.[a]




| Entry | Substrate | Product | % Relative activity[b] | *e.e.* **16**[c] (conf.) | *e.e.* Pure PAMO[d] (conf.) |
|---|---|---|---|---|---|
| 1 | **17** | **18** | 28 | >99% (*S*) | >99% (*S*) |
| 2 | **19** | **20** | 11 | 33% (*R*) | 44% (*R*) |
| 3 | **21** | **22** | 58 | 91% (*S*) | 94% (*S*) |
| 4 | **23** | **24** | 69 | >99% (*R*) | 80% (*R*) |

[a] For reaction conditions, see Section 2.
[b] In comparison with model substrate **14**. Measured by GC.
[c] Measured by chiral GC or HPLC, see Supplementary Data.
[d] Obtained for isolated PAMO as previously described [33,37].

after five catalytic cycles, but an appreciable loss of activity was noticed in the second cycle, and later a constant decrease in the activity of these biocatalysts. It is important to note that a very similar trend was obtained for both polyphosphazenes, independently if fresh G6PDH (for **11**) or not (for **16**) was added in the reaction medium. This seems to indicate that the loss of activity is mainly due to PAMO and not due to G6PDH, which is very stable under these conditions. In a parallel test, purified PAMO was tested against phenylacetone, measuring the enzymatic conversion at 30 min without previous treatment (80%) or after 24 h of incubation in Tris–HCl buffer 50 mM pH 9 at 30 °C (6%). As can be seen, PAMO is clearly inactivated, what supports the assumption that it is the enzyme that can be more sensitive.

Finally, we were interested in studying the immobilization effect on PAMO selectivity performing oxidations on a racemic ketone (**17**) and on prochiral sulfides (**19**, **21**, and **23**). Co-immobilized PAMO and G6PDH biocatalyst **16** was employed (Table 1), resulting in a very similar selectivity to that obtained for purified PAMO [33,37], except for the case of sulfide **19**, where a slight decrease in the stereoselective oxidation process was observed. Interestingly, in the case of sulfide **23**, a remarkable improvement on the selectivity was observed.

## 4. Conclusions

The amino polyphosphazenes $\{NP[O_2C_{12}H_{8-x}(NH_2)_x]\}_n$ (**5–7**), prepared by nitration followed by a reduction protocol starting from precursor $[NP(OC_{12}H_8)]_n$, have been successfully used to covalently immobilize for the first time a BVMO catalyst (PAMO) through glutaraldehyde connectors. As the enzyme works in aqueous solution, its attachment to a solid support presents several advantages for the recycling performance. After optimization of the temperature, reaction time and type of shaking, a good catalytic activity for these immobilized preparations was obtained with a proportion of 0.5 amino groups per monomeric unit ($x = 0.5$). The same enzymatic activity was observed with $x = 1$ or 2. It was also found that the cofactor recycling enzyme G6PDH could be attached on the same polyphosphazene carrier **5** together with PAMO to obtain a synthetic 'self-sufficient' redox biocatalyst. The good stability observed for the recycling enzyme G6PDH on this type of support is very promising to design immobilized biocatalysts to be used in a 'coupled enzyme' approach [45] with other oxidoreductases. This bifunctionalized preparation did not significantly affect PAMO stereoselectivity, and was tried to reuse, although probably due to the lower stability of the monooxygenase, these preparations lost their activity in a great extent after few cycles.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.10.002.

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