Polyphosphazenes as Tunable and Recyclable Supports To Immobilize Alcohol Dehydrogenases and Lipases: Synthesis, Catalytic Activity, and Recycling Efficiency

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The polyphosphazene $\{NP[O_2C_{12}H_{7.5}(NH_2)_{0.5}]\}_n$, prepared by reacting $\{NP[O_2C_{12}H_{7.5}(NO_2)_{0.5}]\}$ with the Lalancette's reagent, was used for attaching enzymes such as alcohol dehydrogenase (ADH-A) and lipase (CAL-B). The resulting new biocatalysts exhibited great potential as tunable supports for enzymatic reactions in both aqueous and organic media. The material with immobilized ADH-A was as efficient as the commercial enzyme to perform stereoselective bioreductions of ketones in aqueous solutions and could be used for the reduction of various aliphatic and aromatic ketones up to 60 °C and recycled several times without significant loss of activity even after three months of storage. The biocatalyst obtained with CAL-B was more efficient than the free enzyme for kinetic resolutions in organic solvents and exhibited a moderately good capability of reutilization.

Introduction

Polyphosphazenes are ideal compounds to tune their physical and chemical properties by selecting the appropriate functionalities linked to the phosphorus atom.[1] Because of their highly valuable characteristics, mainly their chemical and temperature resistance, these derivatives are potentially useful for biotechnological applications and, in fact, novel materials derived from this type of polymer have been recently designed and applied to important fields such as biomedicine.[2] 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.[1,3] The latter is a more advantageous approach in cases like the sulfonation of aryloxyphosphazenes[4] or the introduction of pendant PPh_2 ligands.[5]

In the past few years, an increasing number of catalytic processes for industry have been described. The new requirements that society demands concerning greener and safer methodologies, make the immobilization of (bio)catalysts a very important issue aiming to better recycling efficiency and more economical and environmental friendly processes.[6] Thus, the chemical or physical fixation of enzymes on supporting carriers can be achieved by different methods.[7] In this context, polyphosphazenes have scarcely been used as support of biomacromolecules, and in the particular case of biocatalysts, only a few examples can be mentioned.[2b] In a first article, Allcock and Kwon covalently immobilized tripsine and glucose-6-phosphate dehydrogenase on a support of modified $[NP(OPh)_2]_n$ on an alumina carrier.[8] The process consisted of a nitration-reduction protocol to obtain the corresponding polyaminophosphazene derivative that was subsequently activated with glutaraldehyde and then reacted with the enzyme solution. Later, an invertase was supported on spherical particles of $[NP(OCH_2CF_3)_2]_n$, first displacing the trifluoroethoxy moieties with $NaOCH_2CH_2NH_2$ and then anchoring the enzyme, obtaining good activities for this preparation.[9] Finally, an urease was encapsulated on a hydrogel derived from poly[bis(methoxyethoxyethoxy)phosphazene] through irradiation with γ-rays to cross the polymer and trap the biocatalyst.[10]

Biocatalytic reactions have recently gained more relevance due to the mild conditions employed and the highly chemo-, regio-, and stereoselective transformations obtained to synthesize natural products and drugs.[11] Among all available enzymes, probably hydrolases and oxidoreductases are the most explored catalysts.[12] Thus, lipases are a class of hydrolases that have widely been used to achieve kinetic resolutions through hydrolysis, esterification, and aminolysis reactions, among others.[13] *Candida antarctica* lipase B (CAL-B) has been used in organic solvents to perform the kinetic resolution of alcohols and especially amines.[14] In the case of oxidoreductases, alcohol dehydrogenases (ADHs, also called carbonyl reductases) have been employed to catalyze the stereoselective reduction of prochiral ketones with remarkable selectivities.[15] For instance, the nicotinamide adenine dinucleotide (NADH)-dependent alcohol dehydrogenase ADH-A from *Rhodococcus ruber* DSM 44541 has been isolated and overexpressed in *Escherichia coli* and can be applied to the asymmetric reduction of alkyl- and aryl-substituted ketones or diketones.[16]

To improve the stability of lipases in organic solvents, they have been adsorbed/immobilized in many supports. In case of immobilization of ADHs, fewer reports have been shown and most of these processes were based on adsorption techniques. Only very few examples using covalent binding are described.[17] There is only one previous report where ADH-A has been covalently anchored to amino-functionalized materials, including porous glass beads, magnetic particles, and nanodiamonds.[18]

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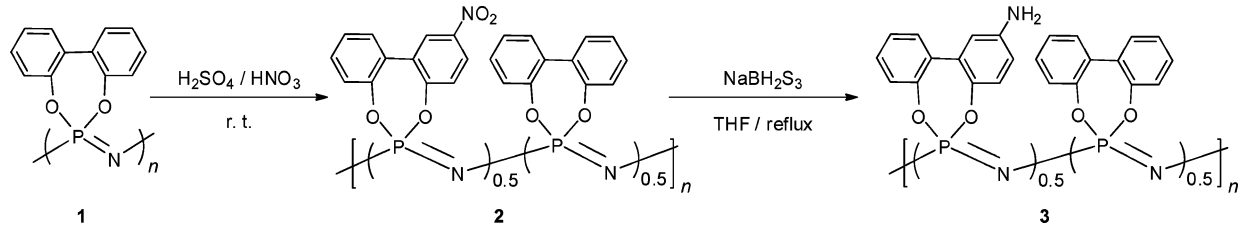
Scheme 1. Synthesis of Polyphosphazene **3**



Glutaraldehyde was employed as linker and, although very good immobilization yields were achieved, the activities were lower. Until three biocatalytic cycles could be performed with a minimum loss of enzymatic activity.

In earlier reports, we described the synthesis of phosphazene polymers with phosphorus-dioxy-biphenyl moieties (or the chiral binaphthyl analogues) in the repeating units, and a number of copolymers with monodentated aryloxydes carrying different functional groups.[19] Recently, the regioselective and controlled direct nitration of the homopolymer $[NP(O_2C_{12}H_8)]_n$ (**1**), which is readily available,[20] was achieved by taking advantage of the unusual high solubility and stability of this precursor in concentrated sulfuric acid, to obtain in one pot a simple procedure for the derivatives $\{NP[O_2C_{12}H_{8-x}(NO_2)_x]\}_n$ (**2**), with x ranging from 0.2 to 2 in excellent yields (Scheme 1).[21] As polymers **2**, with M_w of the order of 10^5, are well characterized materials (by ^{1}H, ^{13}C, ^{31}P, and IR spectra) with well-defined chemical composition, and with limited but sufficient solubility in THF,[21] they were selected as very advantageous candidates to be converted into the NH_2 derivatives $\{NP[O_2C_{12}H_{7.5}(NH_2)_{0.5}]\}_n$ (**3**), useful to support enzymes. Herein we wish to report the use of **3** to immobilize an alcohol dehydrogenase (ADH-A) for bioreductions on aqueous solutions and a lipase (CAL-B) to achieve kinetic resolutions on organic solvents.

Experimental Section

General. Ketones, racemic alcohols, racemic acetate **17**, alcohol dehydrogenase from *Rhodococcus ruber* ADH-A and *Candida antarctica* lipase B were purchased from commercial sources. One unit (U) of ADH-A reduces 1.0 μM of acetophenone to 1-phenylethanol per minute at pH 7.5 and 30 °C in the presence of NADH. Polyphosphazene **1** was synthesized as previously described.[20] Polyphosphazene **2** was synthesized as previously described.[21] 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 ^{1}H and ^{13}C{^{1}H} NMR spectra in deuterated dimethylsulfoxide (DMSO-d^6) are given in δ relative to trimethylsilane (TMS; DMSO at 2.51 and 40.2 ppm, respectively). ^{31}P{^{1}H} 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. T_g 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. The degree of enzymatic conversion corresponded to the measured area of the product in the GC chromatogram among the sum of GC areas of the substrate and product. The enantiomeric excesses were calculated dividing the subtraction of the GC area for the major enantiomer minus the GC area for the minor enantiomer among the sum of the GC areas for both enantiomers.

Synthesis of $\{NP[O_2C_{12}H_{7.5}(NH_2)_{0.5}]\}_n$ (3). First, the Lalancette's reagent[22] was prepared (in a well ventilated hood). Air and humidity must be strictly avoided. A three-necked 250 mL flask, filled with a dried N_2 atmosphere and equipped with a condenser and a bubbler with paraffin oil, was cooled to 0 °C (ice bath). Then the following were added in the order $NaBH_4$ (0.63 g, 16.7 mmol), sulfur (1.78 g, 55 mmol), and recently well-dried and distilled (twice) THF (40 mL; CARE, exothermic reaction with evolution of H_2S). The mixture was stirred for 1 h to give a transparent yellow solution. The volatiles were evaporated in a vacuum, and the residue was washed with diethyl ether (2 × 50 mL) and dried overnight in a vacuum.

To the prepared Lalancette's reagent, THF (50 mL) and a solution of $\{NP[O_2C_{12}H_{7.5}(NO_2)_{0.5}]\}_n$ (**2**, $x = 0.5$; 2 g, 9.96 mmol) in THF (50 mL) were added and the final volume was increased by 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^{-1} aqueous HCl (5 mL) was 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. Yield: 1.51 g (80%). IR (KBr) cm^{-1}: 3435, 3368 m.br (ν NH), 3065 w (ν CH arom.), 1624 br.m, 1502 m, 1478 m (ν CC arom., δ NH), 1384 sh.m (typical of biphenoxyphosphazenes, not assigned), 1347 m, 1266 s, 1247 s, 1191 vs (ν NP), 1096 s (ν P-OC), 1041 w, 1013 w (not assigned), 941−922 s.br (δ POC), 870 w, 819 v.w (not assigned), 785 s, 751 s (δ CH arom.), 607 m, 536 m.br (other); ^{1}H NMR (ppm, DMSO-d^6): 6−8 v.br, (aromatic protons), 4.9 v.br (NH_2); ^{13}C NMR (ppm, DMSO-d^6, all peaks are broad): 151 (C_2), 149 (C_5−NH_2), 132, 131, 128, 125 (C_1, C_3, C_4, and C_6); ^{31}P NMR (ppm, DMSO-d^6): −6.3 br; Anal. (Calcd.): C 57.0 (60.9), 3.40 (3.59), 8.50 (8.87). Sulfur retained 1%; TGA (from ambient to 900 °C): Continuous loss from 300−800 °C. Final residue 44% (under N_2), 7% (under air); DSC: $T_g = 129$ °C, $\Delta C_p = 0.11$ J g^{-1} K^{-1}.

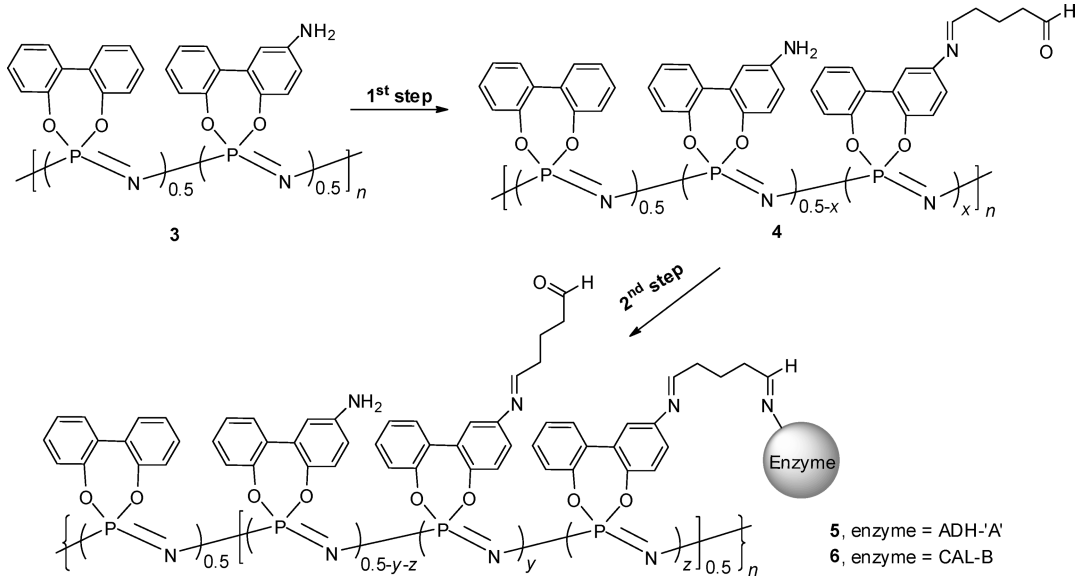
Synthesis of 5 and 6. Polyphosphazene **3** (60 mg) was added to a saturated solution of $(NH_4)_2SO_4$ (9 mL) and a solution of glutaraldehyde (1 mL, 2.5% v v^{-1}) in phosphate buffer (50 mM, pH 7), affording a suspension. Then it was mixed under magnetic stirring for 2 h at 50 °C. The solid obtained was filtered off, washed with phosphate buffer 50 mM pH 7 (3 × 1 mL), and dried under vacuum, affording **4** (61 mg, 87%).

Subsequently, **4** (20 mg) was added to a 1 mL of tris(hydroxymethyl)aminomethane (Tris·HCl) buffer 50 mM pH 7.5 with ADH-A (1 mg) or CAL-B (10 μL), affording a heterogeneous mixture that was magnetically stirred at 5 °C for 15 h. Afterward, the polymer was filtered, washed with Tris·HCl buffer 50 mM pH 7.5 (4 × 0.5 mL), and dried under vacuum, affording **5** or **6** (20 mg).

These formulations were obtained as fine powders, and no control of the particle size (micrometers) could be achieved, so the effects of this parameter in the catalytic activity could not be evaluated.

Enzymatic Reduction of Ketones Employing 5 and 2-Propanol. A total of 20 mg of **5** were placed in an eppendorf tube and then 600 μL of Tris·HCl buffer 50 mM pH 7.5, 1 mM NADH, the corresponding ketone (35 mM), and 2-propanol (32 μL, 5% v v^{-1}) were added. The heterogeneous mixture was shaken under orbital shaking at 30 °C and 250 rpm for 24 h. Afterward, this mixture was extracted with ethyl acetate (2 × 0.6 mL), and the organic phase was dried over Na_2SO_4.

Scheme 2. General Strategy To Covalently Link a Biocatalyst to **3**



Finally, conversion and enantiomeric excess was measured by achiral and chiral GC, respectively.

Study of the Stability of 5 over a 3 Month Period. A total of 20 mg of **5** were placed in an eppendorf tube and then 600 μL of Tris·HCl buffer 50 mM pH 7.5, 1 mM NADH, acetophenone (2.5 μL, 35 mM), and 2-propanol (32 μL, 5% v v^{-1}) were added. The heterogeneous mixture was shaken under orbital shaking at 30 °C and 250 rpm for 24 h. Afterward, this mixture was extracted with ethyl acetate (2 × 0.6 mL), and the organic phase was dried over Na_2SO_4. Finally, conversion was measured by achiral GC. To avoid material loss, the second cycle was performed in the same eppendorf tube, adding to the polymer the rest of reagents, as mentioned before. After 3 consecutive cycles, dry polyphosphazene was stored for 30 days at −20 °C to achieve the following catalytic reactions. To reuse it, the support was simply allowed to warm up to room temperature.

Kinetic Resolution of 1-Phenylethanol Employing 6 and Vinyl Acetate. Compound **6** (20 mg) was introduced in an erlenmeyer flask with dry *tert*-butyl methyl ether (TBME; 0.82 mL). Afterward, 1-phenylethanol (10 mg, 0.08 mmol) and vinyl acetate (22 μL, 0.24 mmol) were added. The heterogeneous mixture was shaken under orbital shaking and kept under nitrogen for 24 h at 30 °C and 250 rpm. Then, the catalyst was filtered and washed with CH_2Cl_2 (2 × 1 mL). Finally, conversion and enantiomeric excesses were measured by achiral and chiral GC, respectively.

Results and Discussion

Synthesis of $\{NP[O_2C_{12}H_{7.5}(NH_2)_{0.5}]\}_n$ (3) by Reduction of 2 (x = 0.5). The synthesis of polymer **3** (Scheme 1) was achieved in 80% yield starting from the readily available polyphosphazene **1**,[20] by selective nitration with a sulfonitric mixture at room temperature during 1.5 h,[21] followed by reduction with Lalancette's reagent[22] under reflux of tetrahydrofuran (see Experimental Section). The composition and structure of the product was confirmed by the analytical and spectroscopic data. The IR showed the expected bands at 3435 and 3368 cm^{-1} (ν NH) and the broadness of the band at 1624 cm^{-1} due to the overlapping with the NH-bending absorption. The presence of the NH_2 group was also observed in the ^{1}H NMR (DMSO-d^6) as a very broad signal centered at 4.9 ppm and also in a weak signal at 149 ppm in the ^{13}C NMR. The low solubility in THF prevented the measurement of the M_w. The TGA (from rt to 900 °C), showed a continuous weight loss from 300−800 °C with a final residue of 44% (under N_2) or 7% (under air). The glass transition was not very clearly observed in the differential scanning calorimetry (DSC) trace (second heating run), but a value T_g = 129 °C (ΔC_p = 0.11 J g^{-1} K^{-1}) could be estimated.

Immobilization of ADH-A on Polyphosphazene 3. To study the use of polymer **3** as support of biocatalysts, the reactivity of the amino groups with the appropriate linkers applied to the subsequent covalent immobilization of a biomacromolecule was considered. Glutaraldehyde, with two reactive aldehyde moieties is one of the connectors more frequently used to achieve covalent unions[6] and, therefore, was first considered as linker between the phosphazene chain and the enzyme (Scheme 2). Because of the low solubility of **3** in most of the solvents tested (H_2O, THF, CH_2Cl_2, and $CHCl_3$) and no swelling was observed in any case, it was also envisaged that the resulting materials could be very appropriate to perform heterogeneous enzymatic transformations in both aqueous and organic media.

In a first approach, we decided to anchor the alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541 ADH-A, known for its high stability and versatility to perform bioreductions on carbonyl groups in aqueous media.[16] Due to the simplicity of reaction conditions, for the first step, we reproduced the methodology described by Liese and co-workers.[18]

Thus, **3** was treated with the linker glutaraldehyde (2 equiv) at room temperature for 2 h. To remove any excess connector, the polymer was washed with buffer. The ^{1}H NMR spectrum of the product in DMSO-d^6, where it was partially soluble, showed the absence of glutaraldehyde. Only when the reaction was performed at 50 °C for 2 h, a singlet at 9.63 ppm was observed showing the formation of **4** (see Supporting Information). The particles of **4** were then reacted with the enzyme solution under magnetic stirring at 4 °C during 30 min, and then the remaining nonreacting aldehyde residues were treated with glycine and the final product was washed with buffer to afford the enzyme attached **5**. The activity of the immobilized biocatalyst in **5** was tested using as a model reaction the stereoselective reduction of acetophenone **7** into 1-(*S*)-phenylethanol **8** in buffer, using 2-propanol to recycle the nicotinamide cofactor in a "substrate-coupled" approach (Scheme 3).[23] The observed enzymatic conversion (13%) was much lower than the obtained with the commercial ADH-A (around 85%),[24] but

Scheme 3. ADH-A-Catalyzed Reduction of Acetophenone in a "Substrate-Coupled" Approach

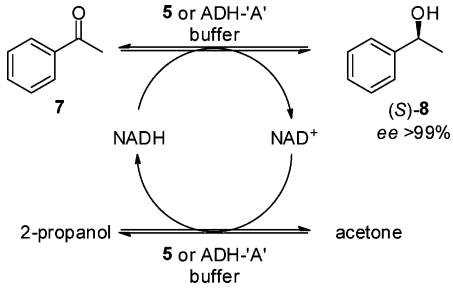


Table 1. Optimization of the Anchoring of Glutaraldehyde to Polyphosphazene **3**[a]

entry	reaction conditions	enzymatic conv.[b,c] (%)
1	initial conditions	13
2	excess glutaraldehyde	12
3	reaction in DMSO	12
4	reaction with Et_3N	17
5	reaction with satd $(NH_4)_2SO_4$	43
6	use of 1,4-diaminobutane as spacer arm	46

[a] For reaction conditions, see Supporting Information. [b] Measured by GC. In all cases, enantiopure (*S*)-**8** was obtained. [c] In all cases, buffer used to wash **5** showed remaining enzymatic activity, demonstrating that ADH-A was employed in excess when was immobilized on polyphosphazene **4**.

the result clearly demonstrated the anchoring of the enzyme to the polyphosphazene.[25] Interestingly, enantiopure (*S*)-**8** was obtained as in the case of commercial ADH-A, showing that selectivity was not affected by the immobilization.

Following these results, the immobilization protocol was optimized. First, the anchoring of glutaraldehyde to **3** was performed by varying several reaction parameters (Table 1). The products of each experiment were equally treated with the enzyme and the glycine solution, and the supported catalysts were tested with the same model reaction (Table 1).

No improvement was observed using an excess of glutaraldehyde (10 equiv) at 50 °C or in the presence of DMSO to make the reaction homogeneous, and only a very slight increase in conversion was noted employing Et_3N as catalyst for the coupling reaction (entries 2−4). However, as in other recent examples,[26] the employment of a saturated aqueous solution of ammonium sulfate (entry 5) afforded a better anchoring of glutaraldehyde as shown by the higher enzymatic activity of the catalyst. These observations can be explained considering that the ammonium salt helps to extend the phosphazene chains facilitating the accessibility of the functionalized sites to the incoming reagents. It is known that reproducible size exclusion chromatograms (SEC) for polyphosphazenes are obtained in the presence of ammonium salts[27] that favor extended conformations.

Table 2. Optimization of the Immobilization of ADH-A on Polyphosphazene **4**[a]

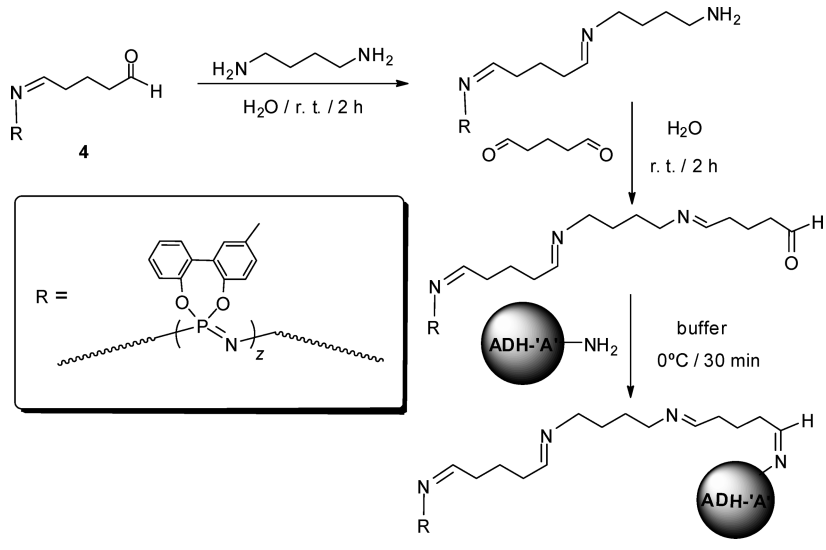
entry	reaction conditions	enzymatic conv.[b] (%)
1	initial conditions	43
2	using satd $(NH_4)_2SO_4$	48
3	reaction time 12 h at 0 °C	85
4	reaction at rt	44
5	orbital shaking at 20 °C	67

[a] For reaction conditions, see Supporting Information. [b] Measured by GC. In all cases, enantiopure (*S*)-**8** was obtained.

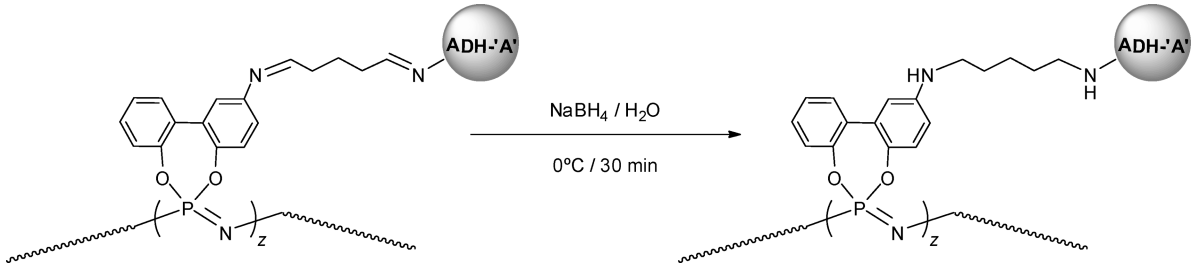
The use of 1,4-diaminobutane as a spacer arm to increase the distance of the reacting groups to the phosphazene main chain, lowering the steric hindrance and minimizing undesirable interactions between the other functional groups on the carrier surface, was also investigated. It has recently been found that immobilized *Candida rugosa* lipase on silica presented a stability improvement using similar linkers.[28] Thus, starting from **4**, a diamine was inserted as shown in Scheme 4. Due to the higher nucleophility of the aliphatic amines, the coupling reactions were done at room temperature. The new biocatalytic preparation showed a good activity (entry 6), but the enhancement was similar to the one achieved with the saturated solution of $(NH_4)_2SO_4$. Therefore, the simpler conditions of entry 5 were chosen as the best ones to perform the first step to the preparation of the supported biocatalyst (Scheme 2). The percentage of glutaraldehyde introduced in the polymer, as estimated by ^1H NMR spectroscopy was around 8% (Scheme 2, approximately $x = 0.04$).

To optimize the enzyme immobilization protocol (second step, Scheme 2), mixtures of polyphosphazene **4** and ADH-A were stirred by varying the reaction conditions, like reaction times, temperature, and type of shaking. The effects on the final activity of the preparations are shown in Table 2.

Scheme 4. Use of 1,4-Diaminobutane as Spacer Arm To Immobilize ADH-A on the Polyphosphazene



Scheme 5. Use of $NaBH_4$ To Reduce the Imine Bonds in Polyphosphazene **5**



Due to the good results obtained for the first step with $(NH_4)_2SO_4$, we stirred a mixture of **4** and ADH-A in a saturated solution of $(NH_4)_2SO_4$ during 30 min at 0 °C, but only a slight improvement was observed (entry 2). However, a great activity enhancement was achieved when ADH-A was mixed in buffer at 0 °C with magnetic stirring during 12 h (entry 3). The activity (85% conv.) was comparable to the one obtained with the commercial ADH-A.[29] The lower conversion observed at room temperature during 12 h with magnetic stirring (entry 4) proved that the immobilization was better performed at low temperature. Because it is known that magnetic stirring can be harmful to enzymes, the orbital shaking at 20 °C was essayed (entry 5). The activity was better in comparison with the anchoring at the same temperature with magnetic stirring, but lower than that obtained at 0 °C. This can be attributed to the fact that when incubating the enzyme directly with the unmodified polyphosphazene **3** using orbital shaking, this led to a preparation with catalytic activity (around 15% conv.), evidencing unspecific adsorptions of the biocatalyst to the polymer.

A final step using a glycine solution to block the nonreacting aldehydes was employed in all cases to obtain the supported catalyst. In an attempt to simplify the protocol, we examined the effects of this step on the enzymatic activity. The ADH-A immobilized on the polyphosphazene without the treatment with glycine exhibited a similar conversion (82%), demonstrating that this step was not necessary.

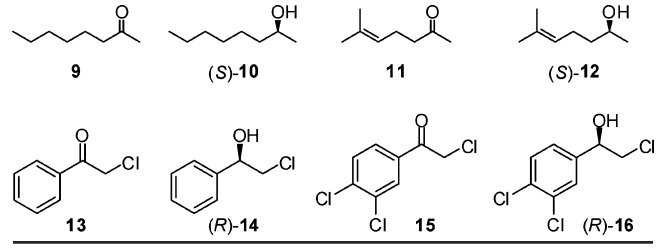
Finally, considering the potential hydrolytic instability of the imine bond present in the catalyst **5** that could diminish its activity within time, a mild reduction agent such as sodium borohydride ($NaBH_4$) was employed (Scheme 5). The material obtained by treating **5** with a solution of $NaBH_4$ in water during 30 min at 0 °C showed a lower enzymatic conversion (62%), suggesting the partial inactivation of the biocatalyst during the reduction. In another experiment, after incubation of **5** in buffer at 30 °C during 24 h, filtration and wash, the buffer used to wash the polymer displayed no enzymatic activity, showing that imine bonds in **5** were stable during its use as catalyst. Therefore, the reduction protocol was not further considered.

As a consequence, the anchoring of ADH-A to polyphosphazene **3** (Scheme 2) was done using the conditions shown in Tables 1 (entry 5) and 2 (entry 3), and no glycine and reduction steps were necessary. Considering that the reaction of the enzyme with the aldehyde is almost quantitative,[18] the final enzymatic preparation possess approximately $y = 0$ and $z = 0.04$ (Scheme 2).

Several ketones were selected to investigate the effects of the immobilization of ADH-A on the catalytic selectivity (Table 3). We observed that aliphatic (**9** and **11**) and aromatic (**13** and **15**) ketones were selectively reduced to the corresponding enantiopure Prelog alcohols with similar conversions as the ones obtained with the commercial ADH-A.

The enzyme immobilization presents several advantages: the protein may become thermally stabilized, allowing its use at higher temperatures,[30] and more importantly, the possibility of recycling exists, therefore, minimizing the cost of the processes. We observed that the enzymatic activity of both commercial ADH-A (already described as a very thermostable enzyme[31]) and **5** remained unchanged after heating in buffer for 72 h at temperatures between 20 and 60 °C (see Supporting Information). Only at 80 °C for 1 h, a loss of 80−90% of the activity was observed in both cases. Therefore, the immobilization of the alcohol dehydrogenase to the polyphosphazene had no positive or negative effect on the thermal stability. Recycling is a key advantage of supported biocatalysts. In the case of ADHs, this is especially beneficial because this enzyme works in aqueous media, where proteins are highly soluble. Therefore, we studied the recycling capacity of **5** applied to the bioreduction of acetophenone. We observed that the immobilized enzyme could be used after six catalytic cycles without appreciable loss of activity (in all cases between 95−100% of remaining activity). We also found that storing the catalyst for 30 days at −20 °C after using it in three consecutive catalytic cycles of reduction of ketone **7**, it could be reutilized to perform three other bioreductions (Figure 1). As can be noted, the immobilized ADH-A was used after 3 months of storage without loss of enzymatic activity, showing the high stability of this preparation and its great potential as biocatalyst for stereoselective bioreductions in aqueous media.

Table 3. Bioreduction of Several Ketones Employing Immobilized ADH-A[a]



entry	ketone	product	enzymatic conv.[b] (%)	e.e.[c,d] (config.)
1	**9**	**10**	78	98% (*S*)
2	**11**	**12**	85	99% (*S*)
3	**13**	**14**	83	99% (*R*)[e]
4	**15**	**16**	99	99% (*R*)[e]

[a] For reaction conditions, see Supporting Information. [b] Measured by GC. [c] Measured by chiral GC. [d] e.e. = enantiomeric excess. [e] Change in Cahn−Ingold−Prelog (CIP) priority.

Immobilization of CAL-B on Polyphosphazene 3. The use of polyphosphazene **3** as an adequate precursor to anchor biocatalysts was extended to other enzymes that work in organic media. Thus, *Candida antarctica* lipase B was selected as a stable enzyme since it has been shown as a very stable catalyst for many selective transformations over secondary alcohols or amines.[14] First, we tried to covalently link CAL-B employing the optimized conditions previously described for ADH-A. This product **6** (Scheme 2) was tested in the acylation reaction of

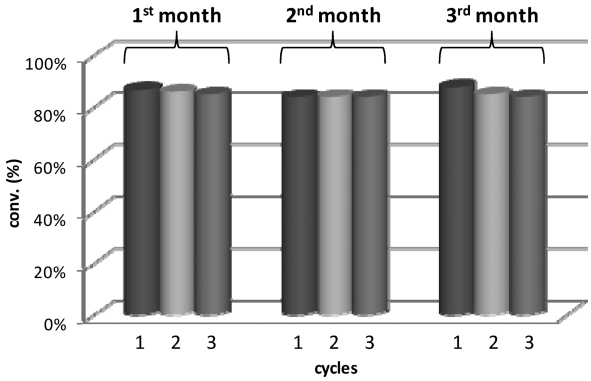
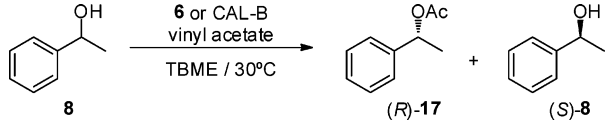


Figure 1. Stability study of **5** vs time in the bioreduction of acetophenone. Three consecutive catalytic cycles were performed and then **5** was kept for 30 days at −20 °C until the next three catalytic cycles were done.

Scheme 6. Enzymatic Acylation of 1-Phenylethanol Using Immobilized CAL-B (**6**) and Vinyl Acetate



rac-1-phenylethanol (**8**) with vinyl acetate (3 equiv) in TBME at 30 °C (Scheme 6) to achieve the kinetic resolution of this substrate. We confirmed that immobilized CAL-B was able to perform this reaction with high selectivity ($c = 49\%$), affording both enantiopure substrate and product after 24 h. Interestingly, under the same conditions, commercial pure CAL-B afforded a conversion of 17%, evidencing an important stability enhancement for this lipase.

To determine the recycling potential of this novel biocatalyst, it was reutilized several times for the kinetic resolution of *rac*-**8** in TBME (Figure 2). The results showed a progressive loss of activity, although, after 5 cycles, the conversions were still higher than 35%.

Conclusions

The novel polyphosphazene derivative $\{NP[O_2C_{12}H_{7.5}(NH_2)_{0.5}]\}_n$, prepared by reduction of the nitro precursor with the Lalancette's reagent, can be advantageously used to synthesize carriers for biocatalysts such as alcohol dehydrogenases and lipases. The covalent attachment of the enzymes was achieved using glutaraldehyde as linker. Several reaction parameters were optimized to obtain good catalytic activities for both preparations. In the case of ADH-A, the catalyst was highly efficient for the stereoselective bioreductions of several aliphatic and aromatic ketones in aqueous media showing a good thermal stability and an excellent recycling capacity, being equally active after three months of storage. For the immobilized CAL-B, this material showed a higher activity than the pure lipase to achieve kinetic resolutions on organic solvents and a moderately good capability of reutilization.

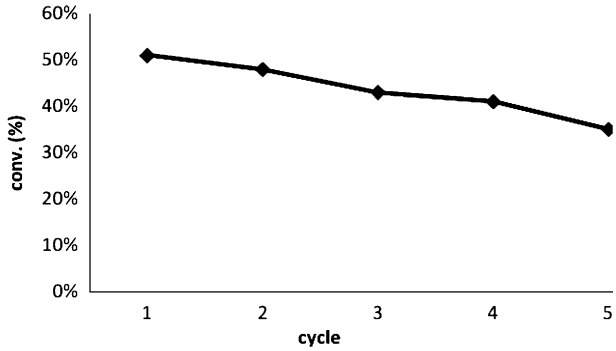


Figure 2. Recycling study of **6** in the lipase-catalyzed acylation of *rac*-**8** ($t = 24$ h).

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Supporting Information Available. Experimental procedures, analytics, and ^{1}H NMR and TGA copies are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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(23) In this approach, an excess of a cheap and easily accessible alcohol such as 2-propanol is employed to force the thermodynamic equilibrium toward the reduction of the desired ketone.
(24) Commercial ADH-A is a powder that completely dissolved in aqueous buffer. To compare the enzymatic activities of both preparations, the quantity equivalent to 10 enzyme units (10 U) was employed in both cases.
(25) To ensure that no unspecific adsorptions occurred, underivatized polyphosphazene **3** was incubated with ADH-A and then was washed with buffer. This carrier did not show measurable enzymatic activity, demonstrating that it came from the biocatalyst covalently linked to the support.
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