Catalytic Saloplastics: Alkaline Phosphatase Immobilized and Stabilized in Compacted Polyelectrolyte Complexes

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Novel biochemically active compact polyelectrolyte complexes (CoPECs) are obtained through a simple coprecipitation and compaction procedure. As shown for the system composed of poly(acrylic acid) (PAA) and poly(allylamine) (PAH) as polyelectrolytes and alkaline phosphatase (ALP) as enzyme, the enzyme can be firmly immobilized into these materials. The ALP not only remains active in these materials, but the matrix also enhances the specific activity of the enzyme while protecting it from deactivation at higher temperature. The presence of the matrix allows fine control and substantial enhancement of reaction rates by varying the salt concentration of the contacting solution or temperature. The excellent reusability, together with the ease of co-immobilizing other useful components, such as magnetic particles, allowing facile handling of the CoPECs, makes these materials interesting candidates for variable scaffolds for the immobilization of enzymes for small- and large-scale enzyme-catalyzed processes.

1. Introduction

Bioactive materials capable of controlling biological processes and systems have attracted much interest over the last years in the fields of biomedical devices, bioremediation, biofuel production and biosynthesis.[1,2] The incorporation or immobilization of enzymes is a particularly versatile way of adding bioactivity to materials. These biocatalytically active materials, which can be employed for drug delivery,[3,4] responsive materials,[5] synthesis,[6,7] elimination,[8,9] and detection of various substances,[10,11] combine the superior specificity of biological processes with the possibility to tailor properties using concepts of materials science.[12–16] Hosts for immobilizing/supporting enzymes, such as organic polymers,[17] biopolymers,[18] hydrogels,[19] and inorganic supports such as porous glass and especially mesoporous silica,[20–23] or carbon nanotubes[24] provide for easy handling and recovery of the often expensive biopolymer. Optimally, these hosts should prevent leaching of the enzyme while allowing substrates to diffuse to the active sites and products to diffuse out into solution.[25] These hosts, which allow one to optimize the activity, stability and specificity of the enzyme,[23] rely on chemical or physical binding of the enzyme to the support or its entrapment in porous structures. More recently, carrier-free systems have been developed,[26] which are obtained by crosslinking enzyme aggregates[27] or enzyme crystals.[28,29]

Complexation of the charged groups of the enzymes with polyelectrolytes can be used as an immobilization technique that avoids the use of chemical crosslinkers, which might interfere with the activity of the enzymes, while the cooperativity of the interactions prevent the enzyme from leaching out of the material.[30] Various types of polyelectrolyte complexes, such as quasisoluble polyelectrolyte complexes[31] and polyelectrolyte multilayers,[32] have been employed for the immobilization of enzymes. Especially the latter technique has been used for their immobilization on supports ranging from hydrogels[33] and porous substrates[23,34] to colloids[35] and for the assembly of enzyme-containing capsules.[36] Macroscopic polyelectrolyte complex materials, which would be of particular interest for large scale applications of immobilized enzymes, were difficult to produce due to the infusibility and insolubility of solution-precipitated polyelectrolyte complexes (PECs).[37–39] Recently, it has been discovered that it is possible to plasticize complexes made of the polyelectrolytes poly(styrene sulfonate) (PSS) and poly(diallyldimethylammonium) (PDADMA) by doping with salt of sufficiently high concentration to allow compaction by ultracentrifugation[40] or extrusion,[41] leading to macroscopically

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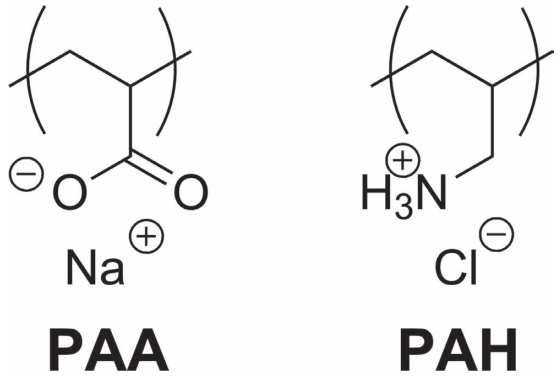
homogeneous materials termed compact polyelectrolyte complexes (CoPECs) with interesting mechanical properties resembling those of cartilage[42] or tendon.[41] This processing technique makes use of the combined effect of small ions (salt) and water to plasticize the complex,[43] giving enough mobility to the polyelectrolyte chains to allow intermixing and fusion at the molecular level and partial expulsion of void water from the diffuse precipitates under the centrifugal field. This densification process yields materials which are tough when wet. Because water is an effective plasticizer the complexes become brittle when dry.[42] More recently, we produced CoPECs of weak polyelectrolytes poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH) (**Scheme 1**) and showed that it is possible to control their composition, porosity, and mechanical properties via the assembly conditions.[44]

Here, we present a new type of material: enzymatically active CoPECs. We show that it is possible to incorporate enzymes during the CoPEC processing and that these enzymes are not only active but that the presence of the polyelectrolytes even enhances their stability and activity. Due to the tunability of the CoPEC properties such materials could become interesting candidates for use in enzyme catalyzed synthesis, bioreactors or waste treatment processes. Furthermore, the CoPECs assembled so far show potential as functional biomaterials, for example, as three dimensional scaffolds for tissue engineering applications. Biomolecules such as enzymes immobilized in these matrices could be used to control the behavior of cells inside these scaffolds.

To illustrate the concept, we immobilized a model enzyme, alkaline phosphatase (ALP), previously deployed in polyelectrolyte multilayers,[45] in PAA/PAH CoPECs. The influence of the assembly procedure on enzyme immobilization and on the activity of the ALP within the complex, as well as the role of diffusion processes, were evaluated. In addition, we studied the stability of the enzyme in the CoPEC over several reaction cycles and against elevated temperature. Finally, we exploited the ease with which other components can be introduced into these blended polyelectrolytes by incorporating magnetic particles along with the enzymes, facilitating handling of these materials.



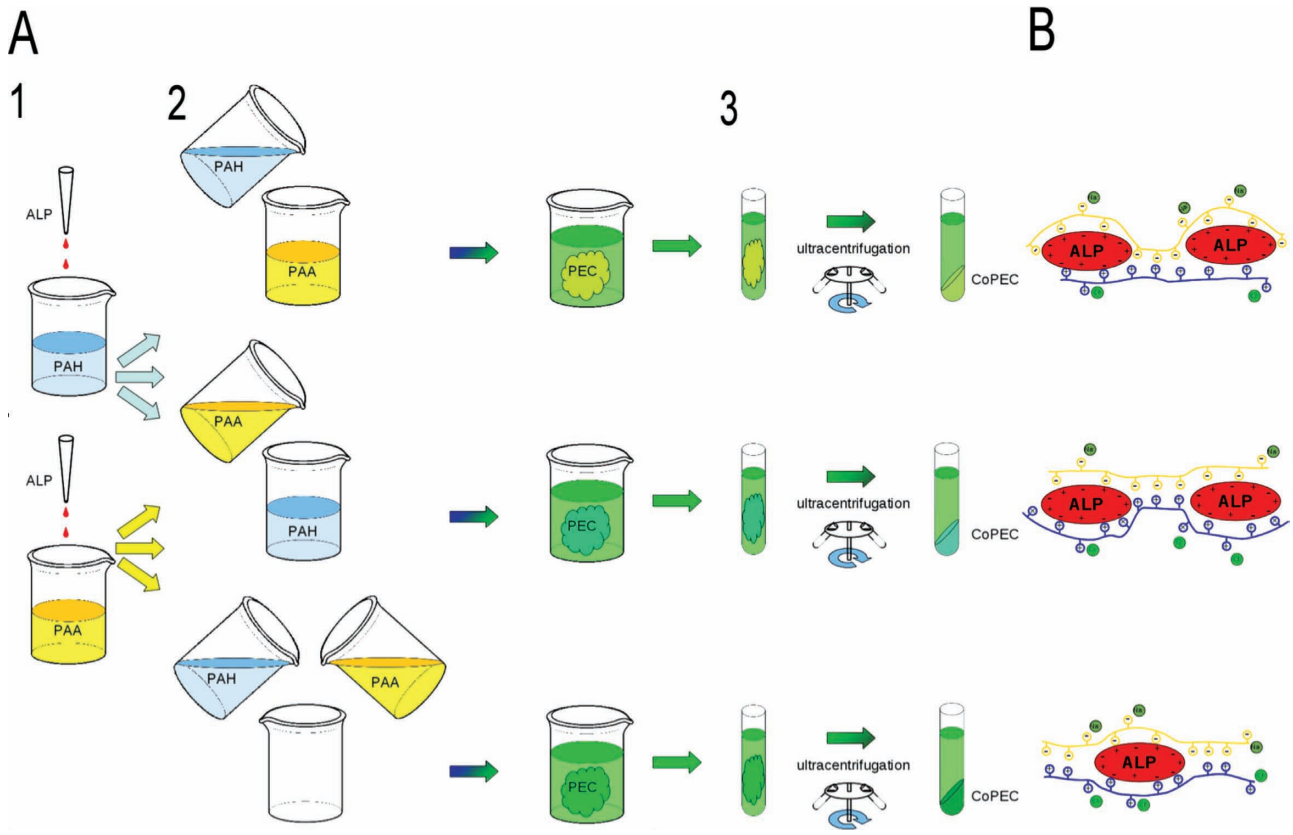
Scheme 1. Structures of poly(acrylic acid) sodium salt (PAA), and of poly(allylamine hydrochloride) (PAH), at pH 7.

2. Results and Discussion

2.1. Immobilization

In a first step we optimized the conditions for immobilization of alkaline phosphatase in compact polyelectrolyte complexes. Interactions of proteins with polyelectrolytes and polyelectrolyte complexes are generally governed by interactions between the charged groups of the polyelectrolytes and charged amino acid residues in the proteins.[46] These interactions are also used for the immobilization of enzymes. Alkaline phosphatase, with an isoelectric point (IEP) of 4.8, has an overall negative charge at the pH of 7.4 used throughout this work. However, both positive and negative charges are present on the enzyme, making charge-charge interactions with both PAA and PAH possible (Scheme 1). Thus we studied different schemes of addition of the three components in order to optimize the immobilization of ALP in the PAA/PAH CoPECs (**Scheme 2** and **Table 1**). First, a solution of ALP was added to a large excess of either PAA or PAH. (Scheme 2, A1). This was thought to lead to association between polyelectrolytes and the enzyme and possibly to the formation of quasi-soluble polyelectrolyte/protein complexes, especially in the case of PAH where a transient clouding at the interface of the two solutions was observed. The enzyme/polyelectrolyte solution was then combined in one of three ways with the other polyelectrolyte (PEL) solution (Scheme 2, A2): i) addition to a beaker containing the other PEL solution, ii) addition of the other PEL solution to a beaker of the ALP-PEL solution, or iii) simultaneous addition of the two PEL solutions to an empty beaker. In all cases the solutions in the beaker were stirred. These different addition schemes allow one to control the ratio of the two polyelectrolytes in the final materials and thus the presence or absence and the sign of the PEL excess charge.[44] Primary complexes typically in the form of powdery precipitates were obtained. Ultracentrifugation of these primary complexes in 1 M NaCl for 4 h led to strong compaction and so to the formation of macroscopically homogeneous, tough materials: CoPECs (Scheme 2, A3). 1 M NaCl was chosen as this provides good plasticization and hence compaction of the complexes, while avoiding denaturation of the enzyme.

To determine the amount of ALP immobilized in these materials we used ALP labeled with rhodamine, ALP^{rho}, in conjunction with fluorescence spectroscopy. The concentration of ALP^{rho} in the supernatant was determined by measuring the fluorescence of the supernate after ultracentrifugation, which provided the percentage of added ALP that had been immobilized in the CoPEC. The results for the different procedures are summarized in Table 1. CoPECs having either an excess of PAH (CoPEC 1 and 2, corresponding to the second row of Scheme 2) or PAA (CoPEC 3, corresponding to the first row of Scheme 2) led to more efficient immobilization of the enzyme than did the stoichiometric CoPEC 4 (corresponding to the last row of Scheme 2), indicating the importance of charge-charge interactions in the final CoPEC. This has previously been observed for quasi soluble polyelectrolyte complexes, where the second polyelectrolyte tended to eject the enzyme.[47] This also explains the relatively low percentage of enzyme



Scheme 2. A) Preparation of PAA/PAH CoPECs containing alkaline phosphatase (ALP): 1) A solution of ALP is added to either PAH or PAA, then 2) combined with a solution of the other polyelectrolyte either by adding PAH to PAA or PAA to PAH, or by adding both solutions simultaneously. The polyelectrolyte complexes (PECs) are then ultracentrifuged (3) to give the corresponding ALP-containing compact polyelectrolyte complexes (CoPECs). B) Depending on the addition order CoPECs contain either an excess of PAA (top) or PAH (middle), or stoichiometric amounts of the polyelectrolytes (bottom). These sequences also influence the amount of ALP immobilized within the CoPECs.

immobilization. Comparing CoPECs 1 and 2 further shows the importance of the mixing order for the immobilization: mixing of the ALP having a negative net charge with the polycation PAH before complexation led to more efficient immobilization than mixing ALP with the polyanion PAA before complex formation. We assume that the stronger interactions between ALP and the polycation favor its immobilization. The CoPECs loaded with ALP^{Rho} were rinsed and then left in 10 mL of a 1 M NaCl solution at pH 7.4. No significant fluorescence was detected in this solution even after 1 week, indicating that no ALP^{Rho} had leached from the CoPEC. Chopping the CoPEC did not lead to a significant increase in fluorescence of the solution either, indicating that the ALP is bound to the matrix and not just trapped in the pores. As enzymes are usually expensive, we performed subsequent studies of ALP-CoPECs with the CoPECs made using the protocol providing the most efficient immobilization.

Table 1. Amount and percentage of alkaline phosphatase immobilized in PAA/PAH CoPECs

CoPEC	ALP mixed with	Order of addition	PAA/PAH ratio of the CoPEC	Immobilized ALP [%]	Mass of ALP per 100 mg of dry CoPEC [mg]
1	PAH	PAA in PAH-ALP	0.9	34 (±2)	0.057
2	PAA	PAA-ALP in PAH	0.9	20 (±2)	0.033
3	PAH	PAH-ALP in PAA	1.1	23 (±2)	0.038
4	PAH	simultaneous	1.0	17 (±3)	0.028

2.2. Activity

We then studied the activity of the ALP immobilized in the CoPEC in order to optimize the CoPEC enzymatic activity. The catalysis of the hydrolysis of the phosphate group of *p*-nitrophenylphosphate (PNPP) by ALP leading to the formation of phosphate and *p*-nitrophenol (PNP) was used for measuring the activity of the enzyme.[48] Since PNP shows a strong absorption peak at around 405 nm, UV/vis-spectroscopy was used to measure the increase of concentration of *p*-nitrophenol with time. The slope of the absorbance–time curve provides the

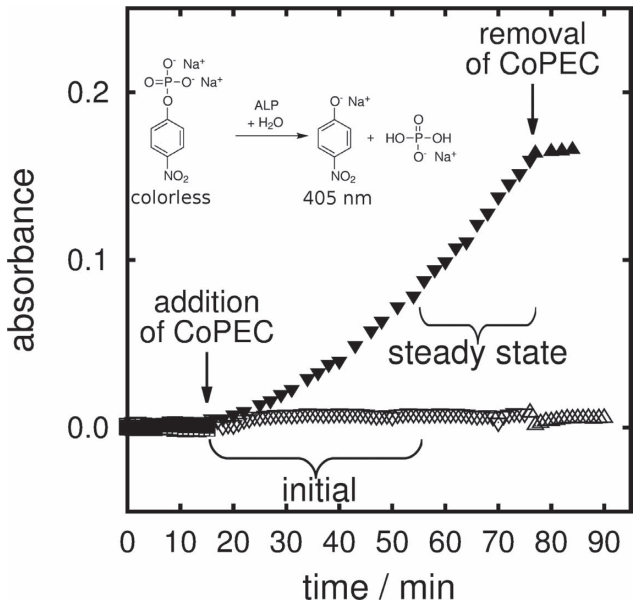


Figure 1. Enzymatic activity of a PAA/PAH CoPEC containing alkaline phosphatase (filled symbols) compared to a PAA/PAH CoPEC without the enzyme (open symbols) at 37 °C. Shown is the absorption at 405 nm, characteristic for the appearance of p-nitrophenol, of a 15 mM solution of p-nitrophenylphosphate as a function of time (reaction shown as inset). After 15 min the CoPEC was added and after 75 min it was removed.

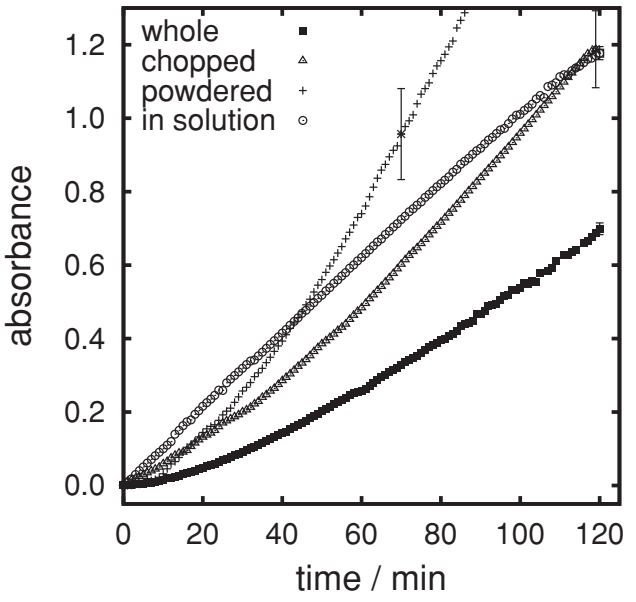


Figure 2. Influence of the dimensions of the CoPEC on enzymatic activity: absorption at 405 nm as a function of time for the whole CoPEC (■), a chopped (▲), and a powdered (+) CoPEC, compared to the same amount (0.06 mg) of alkaline phosphatase in solution (○) at 37 °C. The curves give the average of at least three independent measurements. Error bars show the observed variations at a given point (last point of the curves except for the powdered CoPEC).

activity and, as the mass of enzyme in the CoPEC is known, the specific activity of the ALP in the CoPEC. **Figure 1** shows the absorbance as a function of time for a 50 mM PNPP solution. When a piece of ALP containing CoPEC was added to the substrate solution, the absorbance at 405 nm increased steadily with time, showing the formation of PNP and hence that the ALP in the CoPEC remained active after ultracentrifugation. The slope of the absorbance–time curve increased during the first 45 min following addition (marked as initial in Figure 1) and then became practically constant (steady state). This indicates the importance of diffusional processes for the apparent activity of the ALP in the CoPEC. When the ALP-CoPEC was removed after one hour, the absorbance of the substrate solution remained constant, showing again that no ALP leached into the surrounding solution, and that the catalysis of the PNPP hydrolysis took place within the CoPEC.

Comparing the activity of ALP loaded in CoPEC to the activity of the same amount of ALP in solution (**Figure 2**, filled squares and open circles, respectively) showed that the apparent activity of a given amount of ALP was clearly lower in the CoPEC. However, when the size of the CoPEC was reduced, either by cutting it into small pieces or by lyophilizing and then grinding it, the specific activity increased and even exceeded that found for ALP in solution (dimensions for the whole CoPEC: 2 mm × 12 mm × 12 mm; the chopped CoPEC: 2 mm × 2 mm × 4 mm; the ground CoPEC: 0.5 mm × 0.5 mm × 0.5 mm). Lyophilization alone had no major effect on the activity (see Supporting Information Figure S2). This indicates that the apparent activity of the ALP in the CoPEC is influenced by diffusion, i.e., diffusion of the PNPP into, and the PNP out of, the CoPEC.[25] The higher specific activity of the ALP in the powdered CoPEC, where limitations from diffusion are minimized, might either be due to the matrix leading to a more active form of the enzyme or to interactions of the substrate and products with the charged matrix.[49] In particular, we observed that the presence of PAH can actually increase the specific activity of ALP in solution while the presence of PAA did not have a significant effect (see Supporting Information Figure S3). Furthermore, it is known that PAH interacts strongly with phosphate anions but also that phosphate anions inhibit ALP.[50] The matrix might thus lead to reduced inhibition which could explain the higher specific activity.[20,51] The high specific activity retained by the ALP in the ultracentrifuged complexes also suggests that ultracentrifugation does not compromise the activity of the enzymes.

Because salt concentration has been shown to have tremendous effects on the structure and properties of CoPECs[40,42–44] we studied the influence of salt concentration on the specific activities of ALP either in solution or in the CoPEC. CoPECs were always processed at 1 M NaCl and then brought in contact with solutions of various NaCl concentrations. As seen in **Figure 3**, the effects in solution and in the CoPEC followed the same trend, with the specific activity being highest at the lowest salt concentration. In the case of the ALP immobilized in the CoPEC, one could make use of the dependence of ALP activity on salt concentration to tailor the rate of the reaction or even to sense the surrounding salt concentration. In contrast to ALP in solution, changes of the salt concentration can be performed in situ in the case of ALP immobilized in the CoPEC.

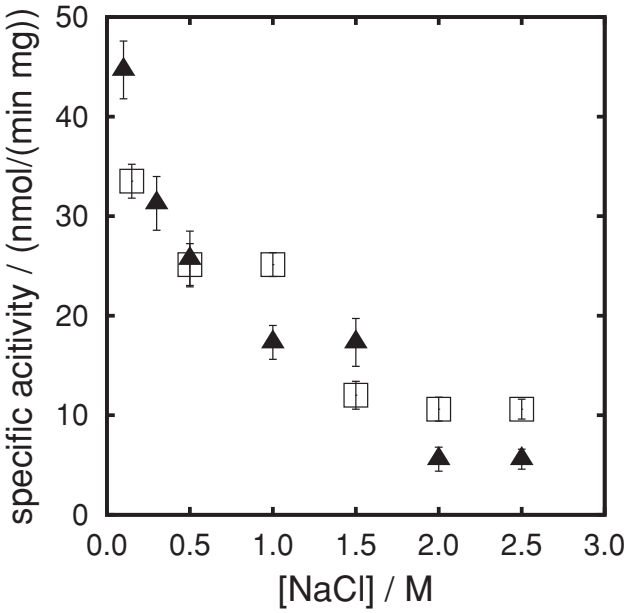


Figure 3. Influence of the salt concentration on the specific activity of alkaline phosphatase in the CoPEC (▲) and in solution (□) measured at 37 °C.

We then studied the reusability of the ALP-loaded CoPECs. **Figure 4** shows the absorbance–time curves for five successive runs on one piece of ALP-CoPEC. In order to eliminate sorbed substrate and/or product before each run, the CoPECs were rinsed overnight in a 1 M NaCl solution. The CoPEC showed practically identical behavior over at least five cycles. Immobilization of ALP into CoPECs thus provides materials for enzymatic catalysis that can be employed in a reproducible manner over several cycles.

2.3. Protection

Apart from easy manipulation and reusability, a major interest in immobilization of enzymes is the possibility to increase their stability towards harsh environmental conditions such as high temperature. Heating solutions of ALP to 60°C led to a 50% drop of the activity in about 45 min (**Figure 5**), and after about 5 h the ALP became practically inactive. However, when ALP containing CoPECs were treated in the same way, activity only decreased very slowly. After 5 h the immobilized ALP had still retained more than 80% of its initial activity. Loss of activity of mammalian ALPs upon heating was attributed to the loss of divalent metal ions,[52,53] followed by changes in the secondary, tertiary, and quaternary structure of the enzymes,[54] and thus to deformations of the active site. Embedding the ALP in the CoPECs probably leads to better stabilization of the metal ions at the active sites due to the additional interactions with charged groups on polyelectrolyte chains surrounding the enzyme and hence to stabilization of the active site. Furthermore, the CoPEC structure may have a scaffolding effect leading to further protection of the enzyme structure.[55] Observations on proteins embedded in polyelectrolyte multilayers, another type of polyelectrolyte complexes, indeed showed that the secondary structure of proteins such as fibrinogen was protected from denaturation by temperature.[56]

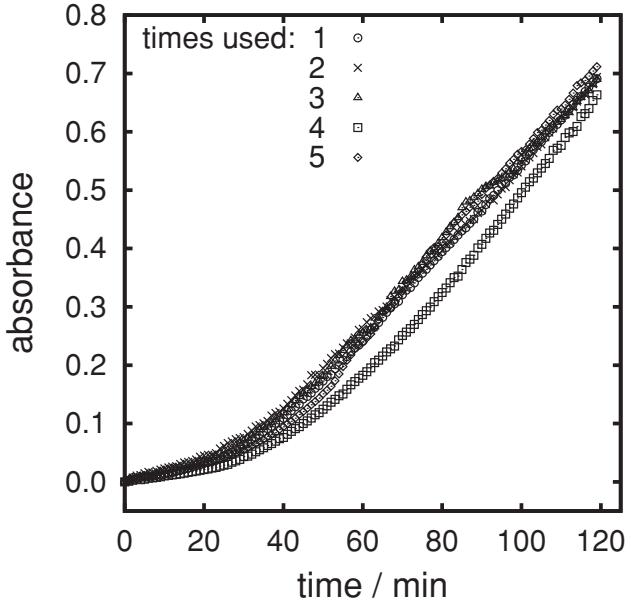


Figure 4. Influence of the number of times a piece of CoPEC was used on the activity of the alkaline phosphatase. Shown is the absorbance at 405 nm as a function of time for several successive enzymatic assays conducted at 37 °C. The CoPEC was rinsed for one night in between two successive experiments.

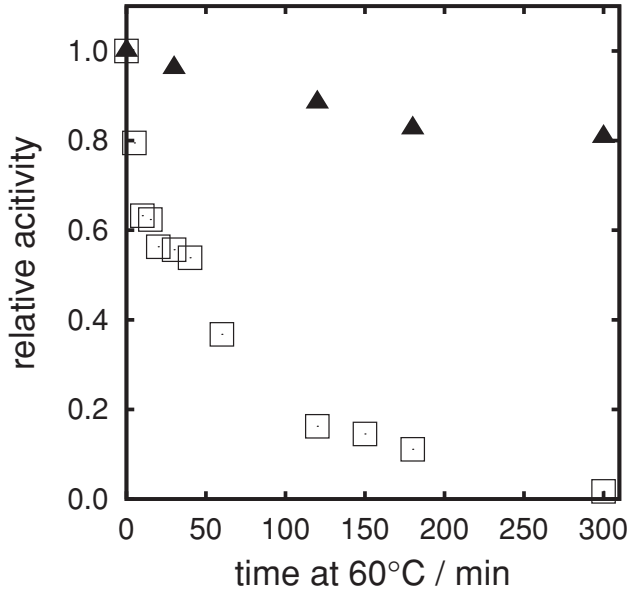


Figure 5. Temperature resistance of alkaline phosphatase in the CoPEC (▲) and in solution (□). Shown are the relative activities (activity at 37 °C without heating set to 1) after different time intervals at 60 °C.

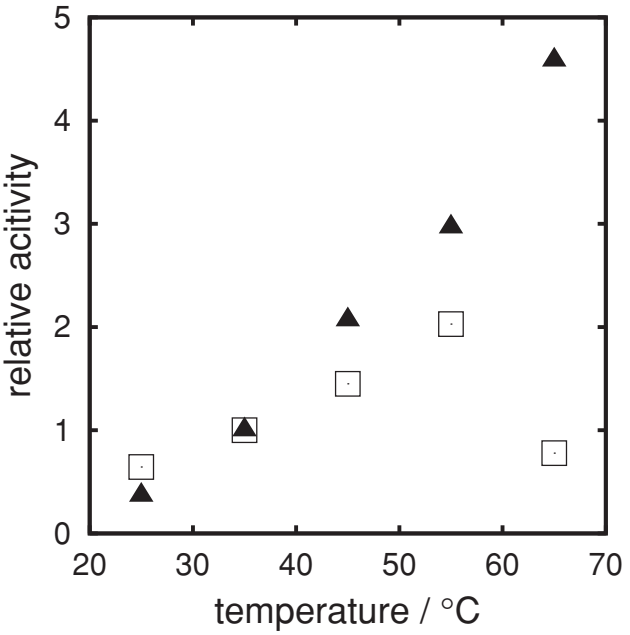


Figure 6. Influence of the temperature on the relative activity of alkaline phosphatase in the CoPEC (▲) and in solution (□). The activity at 35 °C was set to 1.

This higher temperature stability makes it possible to employ higher reaction rates at elevated temperatures.[57,58] We studied the activity of ALP immobilized in the CoPEC (here a whole piece of CoPEC was used) and free in solution at various temperatures. As shown in **Figure 6** the activity of ALP increases as expected with increasing temperature. However, in the case of free ALP in solution a maximum is reached at 55 °C, then activity drops sharply at 65 °C. In the case of ALP immobilized in CoPEC, on the other hand, the activity still increases further, and thus the (specific) activity, even in the case of the whole CoPEC, eventually becomes higher than for the free enzyme in solution. The higher stability of ALP in the CoPEC thus permits higher reaction rates at higher temperatures, providing economic use of enzyme and better control of the reaction rate.

2.4. Magnetic Particles

Automated batch processes using enzyme catalysts would need easy ways of retrieving and handling the immobilized enzyme, such as filtration or magnetic separation. To prepare magnetic CoPECs we used magnetic iron oxide (Fe_3O_4) nanoparticles, which should allow for good dispersion inside the CoPEC matrix. About 1.5 wt% (relative to the dry CoPEC mass) of Fe_3O_4 nanoparticles were introduced into the ALP containing CoPECs by mixing the nanoparticle suspension first with the PAA solution. After precipitation by adding this suspension to an ALP-PAH solution and compaction, dark brown CoPECs were obtained (**Figure 7**). The presence of the magnetic nanoparticles had no significant influence on the specific activity of the ALP immobilized in the CoPECs. However, this small amount of nanoparticles was sufficient to render the materials magnetic and to allow retrieval of the CoPECs from solution using a small magnet.

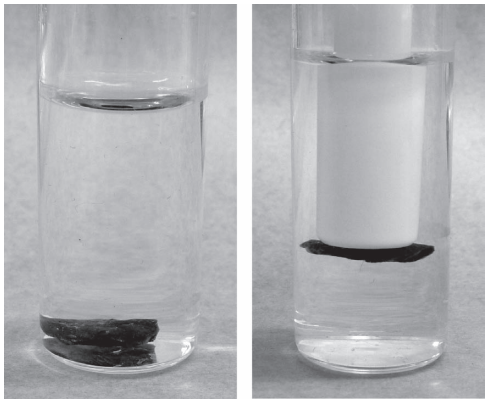


Figure 7. PAA/PAH-ALP CoPECs containing magnetic particles can be retrieved from solution using magnets.

3. Conclusions

It is possible to obtain biochemically active compact polyelectrolyte complexes through a simple coprecipitation and compaction procedure. As shown for the system composed of PAA and PAH as polyelectrolytes and ALP as enzyme, the enzyme can be firmly immobilized into these materials. Furthermore, the ALP not only remained active in these materials, but the matrix actually enhanced the specific activity of the enzyme, while protecting it from deactivation at higher temperature. The presence of the matrix not only allows fine control and substantial enhancement of reaction rates using salt concentration of the surrounding solution or temperature, but also provides materials with enzymatic activities that respond to environmental conditions. The excellent reusability together with the ease of co-immobilizing other useful components, such as magnetic particles, allowing facile handling of the CoPECs, makes these materials interesting candidates for variable scaffolds for the immobilization of enzymes in small- and large-scale enzyme-catalyzed processes.

4. Experimental Section

Materials: Poly(acrylic acid) (PAA, M_w 250 000) and poly(allylamine hydrochloride) (PAH, M_w 58 000) both from Sigma Aldrich, were dissolved in water, dialyzed for 2 days against water, neutralized with 1 M HCl or NaOH, and lyophilized. Human placenta alkaline phosphatase (ALP, lyophilized powder 15 units mg^{-1} and 1 unit mg^{-1}, Sigma Aldrich), rhodamine isothiocyanate (Rho, mixed isomer, Sigma Aldrich), *p*-nitrophenyl phosphate disodium salt hexahydrate (PNPP, 99%, Aldrich), NaCl (>99%, Carlo Erba), tris(hydroxymethyl)-aminomethane (Tris base, 99.96%, Euromedex), DCl (99 at% D, Sigma Aldrich), D_2O (99.9 at% D, Eurisotop), and KBr (>99%, Acros) were used as received. Iron oxide (Fe_3O_4) magnetic nanoparticles (MNPs) (average size: 20–30 nm) were purchased from Alfa Aesar. 18 MOhm MilliQ water (Millipore) was used in all experiments.

Synthesis of ALP Labeled with Rhodamine Isothiocyanate (ALP^{Rho}): ALP^{Rho} was obtained by adding rhodamine isothiocyanate dissolved in dimethylsulfoxide (10 mg mL^{-1}) to a solution of ALP (10 mg mL^{-1}) in 0.1 M Na_2CO_3 solution at pH = 8.5. The molar ratio of rhodamine to enzyme was 10:1. The mixture was stirred at room temperature for 1 h. The product was purified by dialysis for three days (MWCO

12 000–14 000 g mol^{-1}), first against 1 wt% NaCl, then against MilliQ-water, changed three times. After lyophilization ALP^{Rho} was obtained as a red powder in a yield of 90%.

Preparation of PAA/PAH CoPECs Containing Alkaline Phosphatase: PAA and PAH solutions were 0.1 M (with respect to the repeat units) in 10 mM Tris buffer at pH 7.4 containing 1 M NaCl. ALP and ALP^{Rho} solutions were 2 mg mL^{-1} in the same buffer. First, 0.1 mL of the ALP solution was added to 10 mL of either the PAA or the PAH solution and the solution was stirred for 5 min. The primary complexes were then precipitated either by adding one of the polyelectrolyte (or polyelectrolyte-enzyme) solutions to a beaker containing the solution of the other or by adding both solutions simultaneously to one beaker (the different combinations and addition schemes are described in more detail in section 2.1). Additions were performed using a peristaltic pump (Ismatec) with a flow rate of 60 mL min^{-1}. Reactions were stirred with a magnetic stir bar at 200 rpm. The primary complexes, obtained directly after precipitation, were then transferred into polycarbonate thick-wall centrifugation tubes (Beckman Coulter Inc) with their supernatant and ultracentrifuged in a Beckman Coulter Ultracentrifuge using a Ti90 rotor at 188 000*g* for 4 h at 23 °C.

Entrapment of Magnetic Nanoparticles in Alkaline Phosphatase Containing PAA/PAH CoPECs: Iron oxide (Fe_3O_4) nanoparticles were dispersed at 3 mg mL^{-1} in a 10 mM Tris buffer solution at pH 7.4 by sonication for 2h. 2 mL of this dispersion were added into 10 mL of a PAA solution. After mixing for 30 s this solution was added into a PAH-ALP solution and the primary complexes were ultracentrifuged as described above.

NMR Spectroscopy: Proton NMR spectroscopy (Bruker Avance 400 MHz) was used to measure the ratio of PAA to PAH in the complexes as follows: excess solution was removed from a piece of complex (20–30 mg) using paper wipes. To exchange most of the hydration H_2O with D_2O the complex was rinsed with D_2O (in three 0.5 mL aliquots over 8 h). The piece of complex was then dissolved in 0.5 mL of a D_2O solution containing 2.5 M KBr and 0.35 M DCl. The ratio of PAA to PAH in the complex was obtained according to Equation 1 by comparison of the intensity *I* of the signal around 3.35 ppm (corresponding to the hydrogens on the CH_2-group alpha to the amine group) to the remaining signal (corresponding spectra are shown in Supporting Information Figure S1):

$$\frac{PAA}{PAH} = \frac{I_{(1.1-2.9ppm)} - 1.5 \cdot I_{(3.1-3.8ppm)}}{1.5 \cdot I_{(3.1-3.8ppm)}} \quad (1)$$

For calibration, spectra of mixtures of known amounts of PAA and PAH in the same solvent were recorded. Chemical shifts are given relative to the water signal set to 4.79 ppm.

Fluorescence Spectroscopy: Fluorescence spectroscopy using a Fluoromax-4 Horiba-Jobin Yvon spectrometer and the rhodamine labeled ALP was employed to determine the amount of ALP immobilized in the CoPECs. CoPECs were obtained as described above using the ALP^{Rho}. After ultracentrifugation the fluorescence intensity of the supernate was measured (λ_{ex} = 560 nm, λ_{em} = 595 nm). By comparing the results to a calibration curve obtained for known ALP^{Rho} concentrations in the same conditions the concentration of ALP^{Rho} in the supernate, and so the amount of ALP^{Rho} immobilized in the CoPECs, could be determined. Ultracentrifugation of solutions of ALP^{Rho} under the same conditions, but in the absence of polyelectrolytes, did not show significant changes of the fluorescence intensity.

Determination of Enzymatic Activity Using UV-Vis Spectroscopy: UV-vis spectroscopy was used to monitor the enzyme activity. Alkaline phosphatase catalyzes the hydrolysis of primary phosphate esters. As a chromogenic substrate we employed *p*-nitrophenylphosphate (PNPP), which is hydrolyzed to give phosphate and *p*-nitrophenolate (PNP) (Figure 1). The latter shows a strong absorbance at 405 nm, which is used to follow the increase of the *p*-nitrophenolate concentration with time. The measurements were carried out on a Shimadzu 1800 double beam spectrometer thermostatted at 37 °C.

0.3 mL of a 150 mM PNPP solution in 10 mM Tris buffer at pH 7.4 and 2.7 mL of 10 mM Tris buffer at pH 7.4 containing 1 M NaCl were mixed in a cuvette and equilibrated until the absorbance was constant (usually about 5 min). To this solution was added either 0.1 mL of an ALP solution at 0.6 mg mL^{-1} or 230 mg of a CoPEC containing ALP (equilibrated for 1 h in 1 M NaCl at pH 7.4) and the increase in absorbance at 405 nm with time was recorded. The latter was then used to calculate the specific and relative activities of the enzyme for CoPECs containing ALP (or ALP solutions) treated in different ways (see Supporting Information for details on the calculations):

Lyophilization and Grinding: 230 mg of CoPEC containing ALP were rapidly frozen in liquid nitrogen, placed in a round-bottom flask and lyophilized. The dry complex was then either ground using a mortar followed by rehydration in 1 M NaCl and the determination of its enzymatic activity as described above, or the whole CoPEC was rehydrated in 1 M NaCl and the enzymatic activity of the whole piece was measured. For the ground CoPEC the size of the CoPEC particles rehydrated in 1 M NaCl was determined by taking optical micrographs (Nikon inverted microscope, 10× objective) and measuring the dimensions of at least 20 particles using the ImageJ software.

Cutting: In order to obtain larger pieces of CoPEC containing ALP, the CoPEC in its hydrated form was cut into small cuboids using histological blades.

Reusability: The enzymatic activity of a 230 mg piece of CoPEC containing ALP was measured for 120 min as described above. The CoPEC was then rinsed for 22 h in 1 M NaCl at 4 °C to remove substrate and product. Then the enzymatic activity was measured again, and the whole process repeated 5 times.

Temperature Stability: For testing the temperature stability of ALP in the CoPECs relative to that in solution, 230 mg pieces of CoPEC containing ALP or the same amount of ALP in solution were heated for different intervals of time to 60 °C in 2.7 mL of 10 mM Tris buffer at pH 7.4 containing 1 M NaCl. After letting the samples equilibrate at 37 °C for 10 min, the substrate solution was added and the enzymatic activity was determined as described above.

Temperature Dependence of Activity: A piece of CoPEC containing 0.06 mg ALP (1 unit mg^{-1}), or the same amount of free ALP, was added to a solution of the substrate as described above but at 25 °C. After 1 h of equilibration the absorbance at 405 nm was measured as a function of time. After 20 min the temperature was increased to 35 °C, at which the sample was allowed to equilibrate for 20 min before the absorbance was measured as before for 20 min. This process was continued with the same samples at 45, 55, and 65 °C. Specific activities were obtained from the slope of the absorbance vs time curve for the last 10 min of each temperature step.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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