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Development and application of enzyme-metal affinity complex and transition metal catalysts

PhD Thesis Book

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1. Introduction and aims

Nowadays, various catalytic organic chemical reactions are becoming more and more important. Whether chemical or biocatalysis, they typically allow the conversion of the desired compound under much more favorable conditions. Catalytic routes allow to use lower temperatures, less reagents, or even to avoid strong oxidizing or reducing agents.
Using biocatalysts with originally defined chirality, we have the possibility to carry out stereo-, regio- and enantiomeric-selective reactions. Many transition metals can be used in elemental or ionic form during oxidation or reduction processes and several carbon-carbon, or carbon-heteroatom coupling reactions. Metal catalysts can be produced in nano-size with more and more efficient properties and better catalytic activity.
For both enzymes and nanoscale metal catalysts, immobilization on solid supports it's essential for efficient use. Immobilization can increase the stability of the catalyst and at the same time make it easily separable and recyclable.
My PhD thesis focused on the development selective enzyme immobilization technologies based on immobilized metal ion affinity chromatography (IMAC). The aim of our experiments was to show the strong influencing effect of the metal ions on both the immobilization process and the properties of the biocatalyst. To create covalently stabilized biocatalysts two immobilization method were developed. A mixed amino groups and metal chelate groups contained silica nanoparticles were used for a two-step immobilization of histidine tagged phosphatases when different crosslinkers were used for the covalent stabilization. The two-step selective complexation and covalent immobilization of His-tagged enzyme was carried out one-pot via using epoxy-chelate supports. The surface modification method developed by us gives the opportunity to use various complexing agents and multiepoxide compounds during the surface modification of both porous macro-sized beads and nanoparticles.
Gold nanoparticles and ruthenium-chloride as an effective reducing and oxidizing catalysts were also examined in my PhD work. The effect of agarose, a natural polymer rarely used in the production of gold nanoparticles, on the properties of nanoparticles was investigated. We also aimed to stabilize the particles via immobilization on porous methacrylate beads. Finally, the preparation of *N*-substituted *meso*-3,4-pyrrolidines without using of toxic osmium tetroxide catalyst was our last goal. We wanted to develop a reaction pathway with ruthenium chloride.

2. Background

2.1. Enzyme immobilization

The expansion of employing enzymes in food and pharmaceutical industry, development of novel biosensors and synthesis of small organic compounds spawned the need of creating more stable and easily recyclable biocatalysts. Enzyme immobilization can enhance resistance against the conditions of use (pH, temperature, organic solvents) and give an opportunity for reuse and application in continuous flow mode[1]. There are several shortcut possibilities from the cell or enzyme production until

[1] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, *Enzyme Microb.*

reaching the final functional form of the biocatalyst. Choosing the right technique of immobilization depends on the desired use of the biocatalyst, considering the activity, selectivity, stability and economic points of view. Nowadays there are several well-known methods for enzyme immobilization: covalent immobilization, adsorption, embedding in polymer or silica-based matrices[2]. New methods have emerged as well over the last decades, in which specifically tagged enzymes can be immobilized via specific interactions[3].

2.2. Immobilized metal ion affinity chromatography (IMAC)

If a partially purified or a homogenous enzyme is needed, further purification steps have to be accomplished. Recombinant production of proteins allows fusing various affinity tags to the target enzyme and using the most efficient affinity chromatographic methods during the downstream processes.

In the late 1970s, Porath and coworkers discovered that histidine, cysteine and tryptophan amino acids can coordinate with transitional metal ions[4]. They created an agarose gel with iminodiacetic acid complexed to Zn^{2+} and Cu^{2+} ions. This metal ion-charged agarose gel was used successfully to separate human serum proteins. Originally, this phenomenon called immobilized metal ion affinity chromatography (IMAC), is used consciously for protein purification. Histidine tags artificially attached to the recombinant target proteins make them suitable to form stable metal-protein complexes, hence facilitating a simple and effective way for protein purification[5].

2.3. Selective enzyme immobilization methods

IMAC (and the other listed methods) are applicable not only for protein purification but are also useful for selective and oriented enzyme immobilization. This process, when applied without elution of the bound protein from the support, enables selective immobilization of the biocatalyst in one-step. The most used metal complexing agents in this IMAC based selective enzyme immobilization methods are the iminodiacetic acid and nitrilotriacetic acid[6]. Because the immobilization in such case is based on reversible interaction between the fixed metal ion and the protein, in case of enzyme inactivation the exhausted enzyme can be eluted from the surface and the support can be recharged with fresh enzyme in a new immobilization cycle[7].

Technol. **2007**, *40*, 1451.

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[6] G. Cao, J. Gao, L. Zhou, Z. Huang, Y. He, M. Zhu, Y. Jiang, *Biochem. Eng. J.* **2017**, *128*, 116–125; Y.M. Ko, C.I. Chen, C.C. Lin, S.C. Kan, C.Z. Zang, C.W. Yeh, W.F. Chang, C.J. Shieh, Y.C. Liu, *Biochem. Eng. J.* **2013**, *79*, 200–205.

[7] A.K. Vahidi, Y. Yang, T.P.N. Ngo, Z. Li, *ACS Catal.* **2015**, *5*, 3157–3161

Although the use of this complex formation for enzyme immobilization is a very straightforward and rapid way to obtain biocatalysts, but sometimes the conditions of the target biotransformation require more stable immobilization. A good strategy may be to stabilize the complexed enzyme after binding to the IMAC support by other methods. When a covalently immobilized biocatalyst is needed in a single operation process without breaking off the procedure between the purification and stabilization steps, the chelating and covalent binding functions must be present in the same time on the support. A selective and stabile process can be achieved by these strategies if a first rapid complexation is followed by a slower covalent binding step [8].

2.4. Synthesis and application of gold nanoparticles

While gold is known to be inert in everyday life many of its properties change significantly as the size decreases. In the colloidal size range, their reactivity also increases, thus becoming effective catalysts. Nowadays, gold nanoparticles (AuNPs) are being extensively used as efficient redox catalyst materials [9]. The most important model reaction for assessing the catalytic activity of gold nanoparticles is the reduction of 4-nitrophenol by sodium tetrahydridoborate. According to the Langmuir-Hinshelwood model the coordination of the hydride species to the gold surface competes with that of the nitroaromatic compounds to the same catalytic site. Among the putative mechanisms a direct and a condensation pathway were distinguished[10].
One of the colloidal deposition methods developed by *Turkevich* et al. for creating AuNPs by treating hydrogen tetrachloroaurate ($HAuCl_4$) with citric acid in water, where the citrate acts as both reducing and stabilizing agent[11].

2.5 Synthesis of *meso*-diols via oxidation of olefins

The most common method to form *meso*-3,4-dihydroxypyrrolidines applies reaction of 3-pyrroline with *N*-methylmorpholine *N*-oxide and catalytic amount of osmium tetroxide[12]. Although osmium tetroxide is a selective catalyst for *cis*-dihydroxylation, its use is inconvenient due to its high price and toxicity. Although transition metal-free methods exist, in most cases they do not provide the pure *cis* compound[13]. Use of ruthenium catalysts has been reported already in *cis*-dihydroxylations of olefins to produce *cis*-diols selectively in rapid reactions with medium to good yields. The side reactions could be suppressed by addition of *Lewis acids* to the system to promote the hydrolysis of the Ru-diester. A study on the effects of various *Lewis acids* showed that

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$CeCl_3$ as additive in the dihydroxylations of olefins resulted in high yields and the best diol-aldehyde ratio[14].

3. Experimental session

3.1. Immobilization of phenylalanine ammonia-lyase in metal complexes

To the metal ion-complexed resins (20 mg) was added the lysate of *E. coli* cells (30 mg/mL total protein in lysis buffer: 50 mmol L^{-1}; TRIS 150 mmol L^{-1}, pH 8.0) expressing His-tagged *Pc*PAL (4 mL, *Pc*PAL concentration 1.4 mg/mL, sufficient for maximal enzyme loading). The suspension was shaken at 450 rpm for 30 min at room temperature to bind the His-tagged enzyme. The nonspecifically adsorbed host proteins were eluted with sequential addition of the following solutions (1 mL each): low salt buffer (30 mmol L^{-1} KCl; 50 mmol L^{-1} HEPES, pH 7.5); high salt buffer (300 mmol L^{-1} KCl, 50 mmol L^{-1} HEPES, pH 7.5); low concentration imidazole buffer (25 mmol L^{-1} imidazole, 30 mmol L^{-1} KCl, 50 mmol L^{-1} HEPES, pH 7.5). The immobilized biocatalysts were washed with deionized water (3×1 mL) and dried in a vacuum drying chamber at room temperature for 2 h (until the vacuum level dropped below 10 mbar).

3.2. Selective covalent immobilization of phenylalanine ammonia-lyase

The crude cell lysate or the purified enzyme solution was defrosted and centrifuged (3500 rpm, 2 min, 15°C). The supernatant protein solution (1 mL) was added to the actual metal-charged bifunctional support (P:10 mg, MNP:5 mg). The resulted suspensions were shaken for 20 h at room temperature. After the first stage of immobilization, the proteins adsorbed nonspecifically were removed by washing first with low salt buffer (1 mL, 30 mM KCl, 50 mM HEPES, pH 7.5), and then with high salt buffer (1 mL, 300 mM KCl, 50 mM HEPES, pH 7.5) solutions. Next, the non-covalently bonded His-tagged proteins were removed from the cobalt(II)-charged surface by washing with an imidazole solution (1 mL, 500 mM in low salt buffer). Finally, the immobilized *Pc*PAL biocatalysts were washed with TRIS buffer (3×1 mL, 100 mM, pH 8.8) and were tested directly in the ammonia elimination reaction.

3.3. Immobilization of PhoN-Sf on metal chelated silica support

The ASNP-M support (12 mg) was mixed with Tris-HCl buffer (pH 8, 0.25 M, 1 mL) containing crude lysate of phosphatase (1 U×mg^{-1} dry carrier for metal ion, EDTA concentration and bifunctional covalent linking agent screenings; 2 U×mg^{-1} dry carrier for biocatalytic studies). The mixture was shaken at room temperature (120 rpm) overnight. Then the resulted metal-bound phosphatase biocatalysts were washed twice by resuspending in 0.25 M Tris-HCl buffer pH 8 followed by centrifugation (10 min at 3500 rpm). After washing a solution of bifunctional covalent linking agent (2 m/m% final concentration of bisepoxide - GB, NB, PB - or GA) dissolved in 20 v/v% EtOH in 0.25 M Tris-HCl buffer pH 8 was added (1 mL). The mixture was shaken at room temperature (120 rpm) overnight. Then the resulted immobilized phosphatase

[14] B. Plietker, M. Niggemann, *Org. Lett.* **2003**, *5*, 3353; B. Plietker, M. Niggemann, *J. Org. Chem.* **2005**, *70*, 2402.

biocatalyst was washed twice with 0.25 M Tris-HCl buffer pH 8. The immobilized preparations were freeze-dried and stored at 4 °C until application.

3.4. Synthesis, immobilization and application of gold nanoparticles

AuNPs were synthesized using agarose (Type I Agarose) and gold(III) chloride trihydrate at various concentrations of both reagents in water. A solution of agarose (4.5 mL water, 1.0 m/v% final concentrations calculated for the 5 mL overall solution) was heated to 90 °C and vigorously stirred in a heating magnetic stirrer at 400 rpm. When the solution of agarose became transparent (after 15 min of heating), a solution of the gold salt (0.5 mL, 1.0 mM, leading to final concentrations 0.1 mM) was added to the stirred hot solution. After keeping the mixture at 90 °C for 30 min with a continuous stirring, it was allowed to cool down to room temperature to result in a colored hydrogel (AuNPs-AR). After cooling to RT, 400 mg of ethylamine-functionalized macroporous polymer resins (ReliZymeTM EA 403) were added to AuNPs-AR as immobilization carrier. The mixture was homogenized by mechanical stirring, then it was centrifuged for 10 minutes at 330 RCF (RT) to recover the formed AuNPs-AR-P beads. After washing with ethanol, the beads were dried at room temperature. For reduction of **7a–e** in batch mode, AuNPs-AR-P (5 mg) was added to the mixture of **7a–e** (10 mL, 3 mM in H_2O in case of **7a-d** and in case of **7e** in H_2O/ethanol 9/1 v/v) with $NaBH_4$ (2.0 M in triethylene glycol dimethyl ether, 25 fold excess) and the reaction mixtures were shaken at room temperature. Samples were taken after 5, 10, 20, 30, 40, 60, 90, 120 minutes and analyzed by a UV-VIS spectrophotometer measuring the spectra from λ=200 to 600 nm.

3.5. Preparation of *meso*-2,3-dihydroxybutane-1,4-diyl dimethanesulfonate (**14**)

$NaIO_4$ (12.8 g, 60 mmol) and $CeCl_3$ (0.99 g, 4 mmol) were added to distilled water (18 mL) and the mixture was gently heated at 50–60°C until a bright yellow suspension was formed (ca. 10 min). The suspension was cooled down 0–5°C and acetonitrile (50 mL) and ethyl acetate (60 mL) were added to it. After stirring this mixture for 5 min at 0–5°C, a solution of $RuCl_3xH_2O$ (52 mg) in distilled water (1 mL) was added and stirring was continued for 2 min. To the so formed oxidizing mixture was added a solution of (*Z*)-but-2-ene-1,4-diyl dimethanesulfonate (**10**) (9.77 g, 40 mmol) in acetonitrile (10 ml) and the reaction mixture was stirred intensively for 15 min at 0–5°C. The reaction mixture was diluted with ethyl acetate (120 mL) and Na_2SO_4 (20 g) was added. After stirring for 1 min, the solids were filtered out and the organic phase was washed with saturated Na_2SO_3 solution (160 mL). The organic phase was dried over Na_2SO_4 than the solvent was evaporated in vacuum. The residual pale-yellow crystals were recrystallized from dichloromethane (10 mL) to give the desired diol (**14**) as white crystals in 70% yield.

Mp.: 111°C; R_f= 0,33 (hexane:ethyl acetate 2:8); ^{1}H NMR (DMSO-d_6): 3,17 (6H, s, 2 CH_3), 3,64 (2H, s, 2 CH), 4,18 (2H, dd, *J*=10,1, 5,3 Hz, 2 CH-*H*), 4,33 (2H, d, *J*=10,1 Hz, 2 C*H*-H), 5,51 (2H, d, *J*=5,3 Hz, OH); ^{13}C NMR (DMSO-d_6): 36,65 (2 CH_3), 68,62 (2 CH_2), 72,03 (2 CH); IR (KBr): 525, 533, 853, 956, 1080, 1183, 1348, 3036, 3440, 3507.

4. Results

4.1. Reversible immobilization of phenylalanine ammonia-lyase[15]

In this work, we wanted to test the activity and stability of *Pc*PAL enzyme with an *N*-terminal His-tag in its immobilized form as a metal chelate complex. Commercially available polymeric resin with aminoalkyl functions at the surface was modified with dianhydride of ethylenediaminetetraacetic acid (EDTADa) to create a surface capable of complexing transition metal ions (Figure 4.1.). To find an appropriate metal ion for the IMAC-type immobilization five transition metal ion –iron(III), cobalt(II), nickel(II), copper(II), zinc(II)– were tested in the immobilization of *Pc*PAL from crude cell lysate. For each metal ion charged support, maximal enzyme loading was achieved for the biocatalyst.

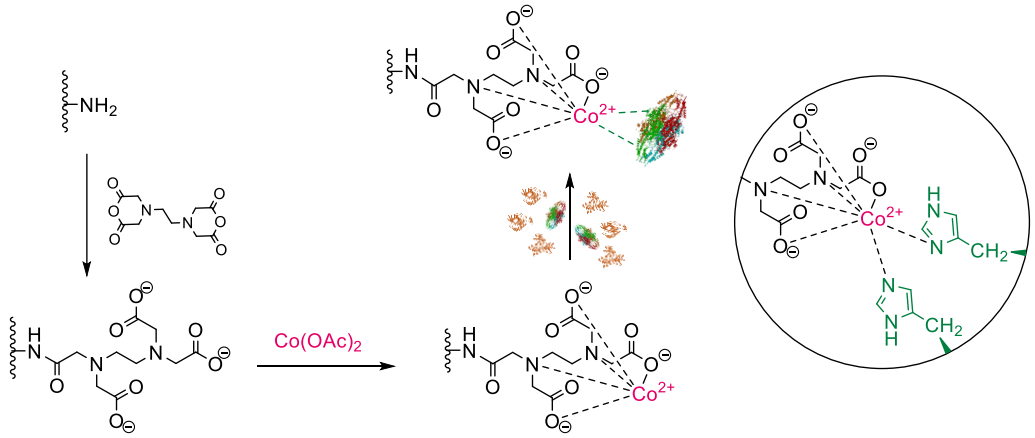


Figure 4.1. Surface modification of amino support for complexation of *Pc*PAL in metal complexes.

The immobilized *Pc*PAL biocatalysts were tested in the ammonia elimination reaction from L-phenylalanine (L-**1a**) leading to *trans*-cinnamic acid (*trans*-**2a**). In case of Fe^{3+} and Cu^{2+} contained supports metal ion leakage was observed. The best biocatalytic activities were observed on the Co^{2+}-charged support (Figure 4.2. A). The composition of the complexed or adsorbed proteins was analyzed with polyacrylamide gel electrophoresis (Figure 4.2. B). According to the SDS-PAGE analysis, the Fe^{3+} ion charged support could complex the target *Pc*PAL with good selectivity but with quite low capacity. Using Co^{2+} and Cu^{2+} ions in the complex resulted in much better capacity, although in the latter case the selectivity was poor due to the easy complexation of amino acid other than histidine to Cu^{2+}. Based on the specific biocatalytic activity, capacity and selectivity results and the simplicity of use and storage, the Co^{2+}-based, dried *Pc*PAL biocatalyst (HA-E_{Co}-PAL) was chosen to characterize further the thermostability and reusability measurements.

[15] D. Weiser, Z, Boros, J. Nagy, G. Hornyánszky, E. Bell, P. Sátorhelyi, L. Poppe, SynBiocat: Protein purification, immobilization and continuous-flow processes. Biocatalysis: An Industrial Perspective, *Cambridge Royal Society of Chemistry Publishing*, **2018**, 397-430. ISBN: 978-1-78262-619-0

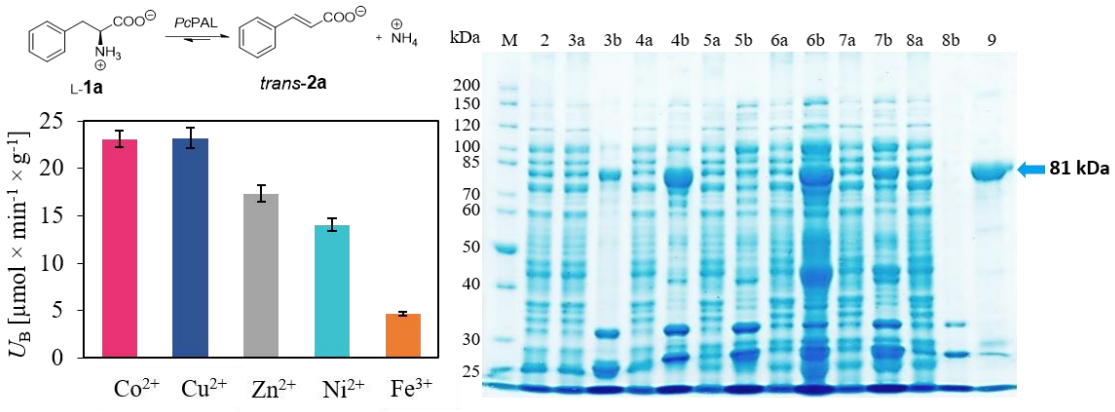


Figure 4.2. A: Specific biocatalytic activity of immobilized *Pc*PAL on chelating supports depending on the used metal ion (■ Co^{2+}; ■ Cu^{2+}; ■ Zn^{2+}; ■ Ni^{2+}; ■ Fe^{3+}). **B:** SDS-PAGE analysis of the immobilized *Pc*PAL biocatalysts. M: Protein ladder, 2: crude lysate of *Pc*PAL, 3-7: *Pc*PAL immobilized on IMAC support charged with various metal ions (3: Fe^{3+}, 4: Co^{2+}, 5: Ni^{2+}, 6: Cu^{2+}, 7: Zn^{2+}, 8: metal free; a: supernatant after complexation, b: polymer after complexation), 9: *Pc*PAL purified on NiNTA.

The thermal behavior of HA-E_{Co}-PAL was measured in 2 hours test reactions between 20 °C and 80 °C. The productivity increased up to 60 °C and then a significant loss of enzyme activity was observed. Our experiments clearly showed that longer incubation at 40 °C or higher temperature caused permanent decrease or complete loss of biocatalytic activity. If temperatures above 65 °C were used, a significant decrease in activity was observed even in the first reaction.

To test the HA-E_{Co}-PAL operational stability at ambient temperature, the biocatalyst was applied in five consecutive ammonia elimination reactions from L-phenylalanine (2 h at 30 °C, each). Although the activity decreased from cycle to cycle, after the fourth cycle 58% of the initial enzyme activity could be retained.

The advantage of enzyme immobilization via affinity binding to metal complexes is the easy reusability of the support after the enzyme losing its activity. To elute the (exhausted) enzyme, a two-step elution was used: at first it was washed with diethlyenetriamine solution (5 V/V%, three times) and after that the remaining proteins were eluted with NaCl (360 g L^{-1}, three times). After the full removal of the enzymes from the previous immobilization, the support was washed with distilled water and then complexed again with Co^{2+} ions. The metal complexed support was used in a new enzyme immobilization cycle. The freshly made and the reused supports were compared in the test reactions as before. Only a very slight difference was observed between the reused and the newly functionalized support (fresh support: 23,1±0,8 U×g^{-1}; regenerated support: 22,8±1,0 U×g^{-1})

By decreasing the surface density of the functional points–in this case the chelating groups–the amount of the immobilized enzyme can be set properly. To modulate the density of the chelating groups on the aminoalkyl functionalized support, glycidol was used in addition to EDTA dianhydride (EDTADa) in different ratios. Beside the creation of IMAC support solely with EDTADa functionalization, modified IMAC supports were also prepared with eight different EDTADa / glycidol molar ratio from 1:1 to 1:36. There was no difference in the specific biocatalytic activity, however, as the ratio increased the amount of immobilized enzyme was decreased. By optimizing the surface, we achieved that the specific enzyme activity of the support modified with

a 1:24 mixture of EDTADa and glycidol (HA-EG24$_{Co}$-PAL) increased by almost 30% compared to the carrier containing only complexing groups (HA-E$_{Co}$-PAL), and achieved the native, free enzyme activity.

4.2. Selective covalent immobilization of phenylalanine ammonia-lyase[16]

The covalent immobilization of *Pc*PAL was implemented on bifunctional epoxy-chelate supports (Figure 4.3.). With the strategy developed by us it was possible to test different multiepoxides to form the covalent binding function and the ratio of chelating and covalent binding sites could be modulated.

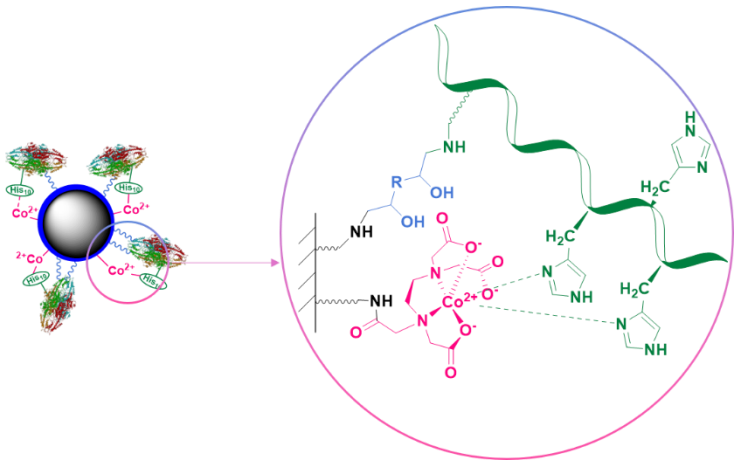


Figure 4.3. Selective immobilization of *Pc*PAL onto mixed epoxy-chelate support.

Determination of the ratio of the bisepoxide compound and ethylenediaminetetraacetic dianhydride (EDa) was performed on a porous polymeric support and also on magnetic nanoparticles. The best specific activity could be achieved when the EDa and bisepoxide (NB) were used in 1 to 75 ratio for polymeric beads and 1 to 10 ratio for magnetic nanoparticles during in the amine-modification reaction (Figure 4.4.).

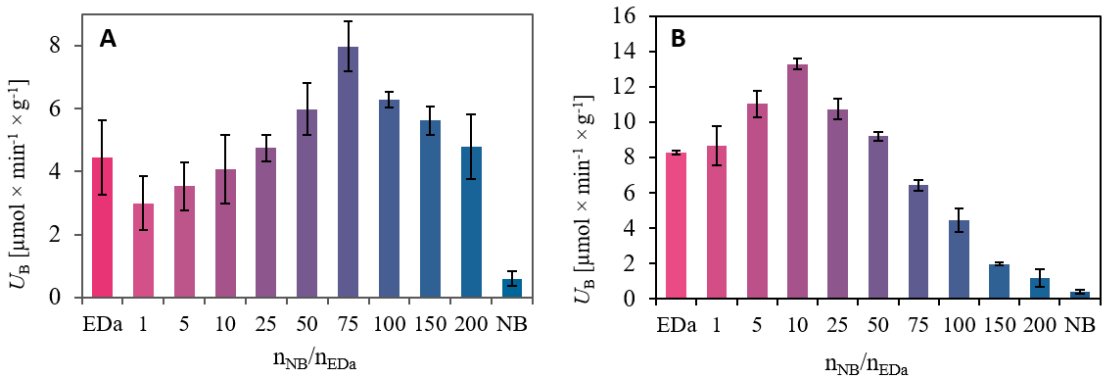


Figure 4.4. Effect of the bisepoxide to ethylenediaminetetraacetic dianhydride ratio in surface functionalization on the activity of the immobilized *Pc*PAL biocatalyst.in the ammonia elimination reaction of L-**1a**. A: polymeric support, B: magnetic nanoparticles.

For both support ten multiepoxides were used for the surface functionalization using the best EDa bisepoxi ratio and the best biocatalytic activities were achieved with the

[16] E. Sánta-Bell, Z. Molnár, A. Varga, F. Nagy, G. Hornyánszky, C. Paizs, D. Balogh-Weiser, L. Poppe: *Molecules*, **2019**, *24*(*22*), 4146.

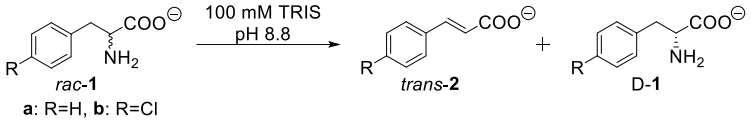
bulkiest trisepoxide containing three aromatic rings (P:14,8 U/g; MNP:24,7 U/g).
To provide guidance in which cases this selective immobilization method is worth using, we investigated the immobilization of *Pc*PAL with the epoxy-chelate MNPs compared to the epoxy only MNPs as support for immobilizations from protein mixtures of eight different target protein contents (Table 4.1). For accurate modeling, known amounts of purified enzyme in lysis buffer were added to cell lysate to mimic mixtures representing different expression levels. The results clearly indicated the benefits of the optimized MNP-E/NP10$_{Co}$ for selective immobilization of *Pc*PAL not just in expression levels below 10 m/m % target enzyme content (>13-fold enhancement in specific activity compared to the non-selective MNPs support) but even in case of 50 m/m % target protein concentration (1.6-fold enhancement).

Table 4.1. Comparison of the efficiency of *Pc*PAL immobilization with epoxy and optimized epoxy-chelate supports from protein mixtures of different target enzyme content. The biocatalytic activity was determined in the ammonia elimination reaction of L-**1a**.

Target protein concentration [m/m%]	$U_B^{\text{MNP-NP-PAL}}$ [μmol/min^{-1} × g^{-1}]	$U_B^{\text{MNP-E/NP10}_{Co}\text{-PAL}}$ [μmol/min^{-1} × g^{-1}]	$\frac{U_B^{\text{MNP-E/NP10}_{Co}\text{-PAL}}}{U_B^{\text{MNP-NP-PAL}}}$
3.8	0.4	13.7	34.2
5.0	0.5	12.3	24.1
10.0	1.0	12.6	13.2
25.0	3.3	13.2	4.0
50.0	8.3	13.4	1.6
75.0	11.9	13.6	1.2
90.0	12.4	13.6	1.1
100.0	14.2	14.1	1.0

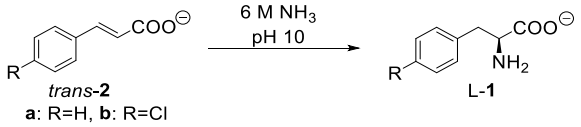
Two immobilized *Pc*PAL biocatalysts were chosen for repeated batch tests: MNP-E/NB10$_{Co}$-PAL and MNP-E/FT10$_{Co}$-PAL The biocatalysts were tested by five repetitive cycles in the ammonia elimination of racemic phenylalanine (*rac*-**1a**) and racemic 4-chlorophenylalanine (*rac*-**1b**) in a traditional buffer system (TRIS, 100 mM, pH 8.8). The conversions and enantiomeric excess values achieved by the immobilized biocatalyst are presented in Table 4.2 Although in the resolution of *rac*-**1a** the activity of both biocatalysts decreased cycle-by-cycle, after five batch reaction the catalysts retained 90% of their initial activities.
The reusability of these biocatalysts was tested also under more demanding conditions in the opposite reaction direction to catalyze ammonia addition onto *trans*-cinnamic acids (*trans*-**2a,b**) producing L-phenylalanine and L-4-chlorophenylalanine. The activity of the MNPs-E/FT10$_{Co}$-PAL was barely diminished during the recycling even of the negative effect of the long-term presence of high ammonia concentration. Both biocatalysts produced L-phenylalanines at high conversion rate with an excellent enantiomeric excess even after five repeated batch reactions (Table 4.3).

Table 4.2. Conversions and enantiomeric excess values of the operational stability tests of immobilized *Pc*PAL biocatalysts in the ammonia elimination reaction of D,L-phenylalanines (*rac*-**1a** and *rac*-**1b**).



	Reaction cycle	MNP-E/NB10$_{Co}$-PAL		MNP-E/FT10$_{Co}$-PAL	
		c [%]	*ee*$_{D\text{-}1}$[%]	*c* [%]	*ee*$_{D\text{-}1}$[%]
rac-**1a**	1	49	82	52	93
	2	48	78	51	92
	3	45	71	48	91
	4	43	67	47	91
	5	43	64	47	89
rac-**1b**	1	49	»99	50	»99
	2	49	»99	50	»99
	3	49	»99	49	»99
	4	49	»99	50	»99
	5	49	99	49	»99

Table 4.3. Conversions and enantiomeric excess values of the operational stability tests of immobilized *Pc*PAL biocatalysts in the ammonia addition onto *trans*-cinnamic acids (*trans*-**1a** and *trans*-**1b**).



	Reaction cycle	MNP-E/NB10$_{Co}$-PAL		MNP-E/FT10$_{Co}$-PAL	
		c [%]	*ee*$_{L\text{-}1}$[%]	*c* [%]	*ee*$_{L\text{-}1}$[%]
trans-**2a**	1	85	»99	86	»99
	2	86	»99	87	»99
	3	85	»99	86	»99
	4	82	»99	86	»99
	5	70	»99	83	»99
trans-**2b**	1	92	»99	91	»99
	2	91	»99	91	»99
	3	88	»99	89	»99
	4	79	»99	85	»99
	5	61	»99	73	»99

4.3. Selective immobilization of acid phosphatases[17]

Experiments on the immobilization of phosphatase enzyme were carried out in collaboration with the Technical University of Graz. I worked on the presented research together with Flóra Nagy.

The aim of our research work was the immobilization of a His-tagged acid phosphatase - Phon-Sf - on bifunctional silica particles containing metal ion complexing groups and amino groups. During the immobilization process, the target enzyme was selectively complexed from the cell lysate in the first step, and then in a second step, covalent

[17] F. Nagy, G. Tasnádi, D. Weiser, E. Bell, M. Hall, K. Ditrich, K. Faber, L. Poppe, *ChemCatChem*, **2018** *53* pp. 1-11.

bonds were formed between the amino groups of the surface and the reactive side chains of the enzyme using bisepoxides or glutaraldehyde. The amount of the complexing agent and the type of the metal ion were investigated (Figure 4.5). The nickel(II) containing particles proved to be the most suitable for acid phosphatase immobilization based on the particles capacity and selectivity, and also the final biocatalytic activity. Covalent stabilization was performed with three bisepoxy compounds (GB, NB, PB) and glutaraldehyde, which is often used for covalent enzyme immobilization (Figure 4.5. C). The highest biocatalytic activity (220 U×g^{-1}) was earned when the neopentyl glycol diglycidyl ether was used for covalent stabilization.

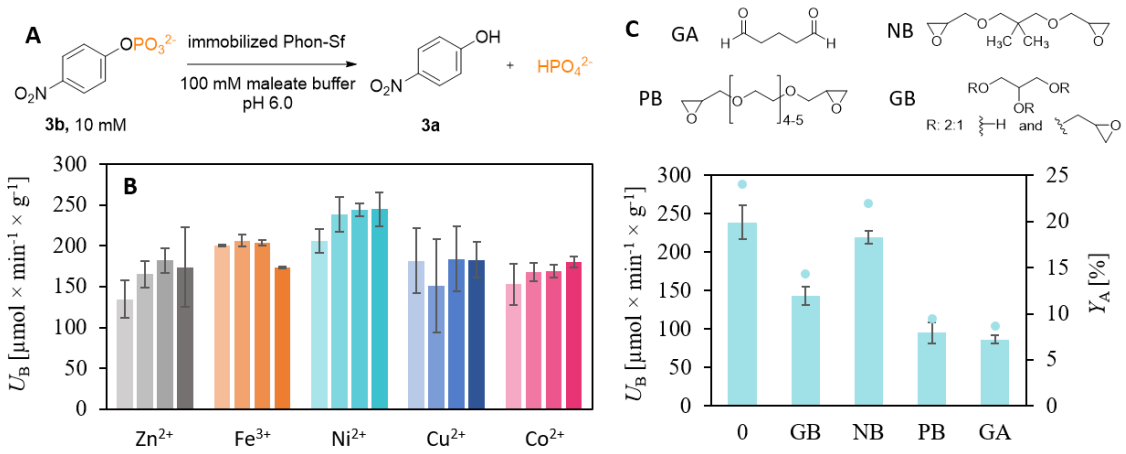


Figure 4.5. Catalytic activity of immobilized Phon-Sf biocatalysts in the hydrolysis of 4-nitrophenyl phosphate (**3b**) (**A**) **B:** Effect of the metal ion and the amount of metal complexing agent on the surface The amount of EDa used in the surface modification were as follows: 0.125; 0.250; 0.375 and 0.500 equivalents calculated based on the amount of aminosilane used on the previous step **C:** Effect of the crosslinker type on the final activity.

Doubling the amount of the enzyme during the immobilization, the final biocatalityc activity was increased almost threefold (600 U×g^{-1}), which is far exceeded the activities obtained by using commercial iron(III) contained supports (EziG-1, 2, and 3Fe) especially developed for immobilization of His-tagged enzymes (80-105 U×g^{-1}).

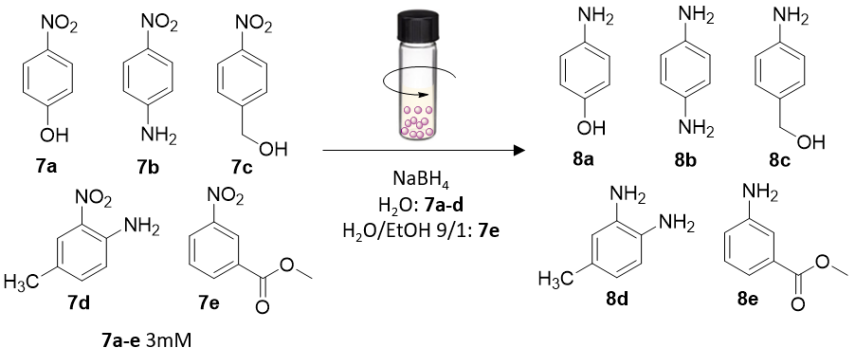
4.4. Synthesis, immobilization and application of gold nanoparticles[18]

The effect of the concentrations of gold salt ($AuHCl_4 \times 3H_2O$) and agarose was systematically studied on the synthesis of AuNPs. After selecting the 0.1 mM $HAuCl_4$ and 1 m/V% agarose concentration for the optimal conditions the synthesized gold nanoparticles entrapped in agarose gel were immobilized on macroporous polymeric beads (Resindion EA 403/S).

The resulting easy-to-handle gold catalyst, which had good mechanical stability, was used in the reduction of 5 aromatic nitro compounds. In the continuous-flow experiments, the AuNPs-AR-P-mediated reduction of *p*-nitrophenol (**7a**) to *p*-aminophenol (**8a**) by sodium borohydride ($NaBH_4$) used in 10-fold excess under alkaline conditions was investigated as a model reaction. As expected, both the

[18] R. Szűcs, D. Balogh-Weiser, E. Sánta-Bell, E. Tóth-Szeles, T. Varga, Z. Kónya, L. Poppe, I. Lagzi, *RSC Advances*, **2019**, *9*, pp. 9193.

concentrations of substrate **7a** and the flow rate had significant impact on the conversion of the *p*-nitrophenol reduction in continuous-flow experiments and conversions between 17 % and 100 % could be achieved.
The effluents investigated in the wavelength region characteristic for AuNPs (λ = 523 nm) indicated no leaching of the catalyst in any of the experiments. By using our flow system, the catalyst is readily reusable after the reaction just by washing the packed bed column with water to remove the lingering reactants.



	r_{sz} [μmol × min^{-1} × g^{-1}]
7a	332 ± 18
7b	312 ± 10
7c	63 ± 5
7d	332 ± 10
7e	67 ± 2

Figure 4.6. Application of Au-AR-P as an immobilized catalyst in reduction of **1a**–**e**.

4.5. Novel synthesis of 1-substituted *meso*-3,4-dihydroxypyrrolidines with a $RuCl_3$-catalyzed key reaction[19]

Since the oxidation of compound **12a** in the presence of ruthenium was not successful, the *cis*-dihydroxylation of dimethanesulfonate **10** in the presence of $RuCl_3$ and $NaIO_4$ was carried out based on previous literature analogy. After optimization of reaction conditions, the desired **14** diol was obtained with 70% yield using 0.005 equivalents of $RuCl_3$ catalyst and no column chromatography is needed. Cyclization was accomplished with three aromatic amines to give the substituted *meso*-3,4-dihydroxypyrrolidine derivatives (Figure 4.7.).

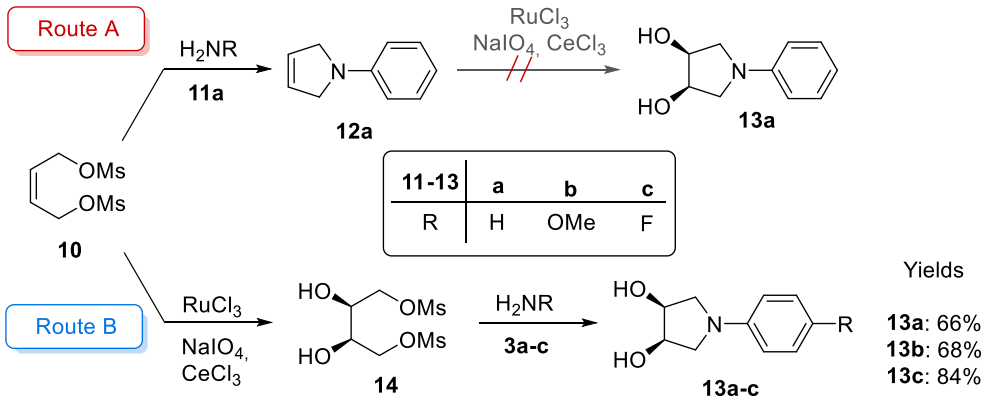


Figure 4.7. Synthetic routes to *meso*-3,4-dihydroxypyrrolidines **13a-c**.

[19] E. Bell, Z. Boros, G. Hornyánszky, *Periodica Polytechnica-Chemical Engineering*, **2015** *59:(2)* 124.

5. Thesis

1. We successfully performed the selective and reversibly immobilization of the phenylalanine ammonia lyase from *Petroselinum crispum* using immobilized metal ion affinity chromatographic support. We found out the highest selectivity, capacity and the most efficient biocatalyst could be earned with the cobalt(II) ion contained support. Specific enzyme activity was maximized by controlling the amount of metal chelate groups using glycidol besides the complexing agent during surface modification. [P I, P III]
2. We developed a regeneration method suitable for removing reversibly immobilized enzyme and metal ion from the surface of the metal chelate support. We demonstrated that immobilization on a regenerated support results in an equally effective biocatalyst as using a freshly surface-modified support. [P I]
3. We developed a new surface modification method for the selective immobilization of histidine-tagged enzymes that is easy to apply to any surfaces functionalized with amino groups and allows the application of a wide range of complexing agents and epoxy compounds. We successfully applied the method and optimized it for the selective immobilization of phenylalanine ammonia-lyase on two different support types. By performing the immobilization with ten different epoxy compounds, we proved that the type of modifying compound has a great impact on the activity of the final biocatalyst. [P II]
4. We proved that the bifunctional epoxy-chelate magnetic nanoparticles developed by us is more efficient in the immobilization processes even at a high target enzyme concentration than the nanoparticles containing only epoxy functions (without a metal ion affinity groups selective for the target enzyme). We successfully performed the resolution of racemic phenylalanine and racemic 4-chlorophenylalanine and prepared L-phenylalanine and L-4-chlorophenylalanine with selectively immobilized phenylalanine ammonia-lyase catalysts. We demonstrated that the PAL catalysts were recycled in five cycles to form L-amino acids with excellent enantiomeric excess in the ammonia addition process. [P II]
5. We performed a two-step selective immobilization of an acid phosphatase enzyme directly from the cell lysate. By systematically optimizing the type of metal ion and the amount of complexing agent, sufficient amino groups remain for subsequent covalent stabilization even at high enzyme binding capacity. Using neopentylglycol diglycidyl ether as a crosslinker, only a little reduction in the specific biocatalytic activity was observed after covalent stabilization. The activity of the immobilized phosphatase biocatalysts we developed far exceeded the activities obtained with commercial carriers used as reference. [P IV]
6. A macroporous methacrylate polymer was effectively used as a solid support to immobilize gold nanoparticles embedded in an agarose gel. We revealed that the size and size distribution of the resulting gold nanoparticles could be well controlled by fine-tuning the concentrations of agarose and gold precursor. We successfully used the thus prepared catalyst in the reduction of five aromatic nitro compounds. We have also demonstrated the benefits of a polymeric support with the application of the catalysts in continuous flow system. [P V]

7. We performed the oxidation of the (Z)-but-2-ene-1,4-diyl dimethanesulfonate in a ruthenium-catalyzed reaction, and formed a *cis*-diol function. The cyclization of the obtained compound was carried out with three aromatic amines. We have developed a much more economical-friendly and safety route to prepare *N*-substituted *meso*-3,4-dihydroxypyrrolidine compounds instead of the very expensive and toxic osmium tetroxide catalysis used previously. [P VI]

6. Summary and potential applications

During my PhD work, our aims were to immobilize and use chemical and biocatalysts and to demonstrate the advantages of immobilization: easy separation, simple recycling, and application in a continuous flow system.

Immobilization of histidine-tagged enzymes was performed in several different ways, in each case using the IMAC technology to provide selectivity. Thus, omitting the costly protein purification procedures, the immobilizations were performed directly from the cell lysate. In addition, the method may also be suitable for the immobilization of histidine-tagged enzymes that are not stable enough and would precipitate or be inactivated during purification procedures. The IMAC method was used for reversible immobilization and also in combination with covalent stabilization methods. An appropriate carrier regeneration protocol was developed to remove the reversibly immobilized enzyme from the metal-protein complex. This provides an opportunity to easily recycle more expensive or difficult-to-produce supports (e.g., biosensors) in case of enzyme inactivation. For selective covalent immobilization a multi-step and also a one-pot method were developed. The advantage of these methods is that it allows an extremely wide variety in the supports, the epoxy compounds and complexing agents used, which has a great effect on the final biocatalytic activity, thus, we can find the most suitable surface for our enzyme.

Using the rarely used natural polymer, agarose, gold nanoparticles were prepared and the effect of agarose and gold precursor concentrations on the properties of the formed particle was investigated. The nanoparticles embedded in the agarose gel was immobilized on a porous polymer support. The efficiency of the catalyst in the reduction of five aromatic nitro compounds was demonstrated in batch mode. The reaction was also carried out in a continuous flow system with the most used model reduction reaction of 4-nitrophenol.

Oxidation of the (Z)-but-2-ene-1,4-diyl dimethanesulfonate was performed in ruthenium-catalyzed reactions and a *cis*-diol structure was formed. The compound thus prepared is cyclized with various amines to give *N*-substituted meso-3,4-dihydroxypyrrolidines, which have previously been prepared mostly by expensive and toxic osmium tetroxide catalysis. Thus, we have developed a much more economical friendly and safety way to prepare these compounds.

7. Publication list

Publication of which the thesis based

P I **Evelin Sánta-Bell**, Norbert Krisztián Kovács, Bálint Alács, Zsófia Molnár, Gábor Hornyánszky: Immobilization of phenylalanine ammonia-lyase via EDTA based metal chelate complexes - optimization and prospects. *Periodica Polytechnica-Chemical Engineering,* **2021,** *accepted*. (SBE: 75%; IF-2018: 1,382)

P II **Evelin Sánta-Bell**, Zsófia Molnár, Andrea Varga, Flóra Nagy, Gábor Hornyánszky, Csaba Paizs, Diána Balogh-Weiser, László Poppe: „Fishing and hunting"– Selective immobilization of a recombinant phenylalanine ammonia-lyase from fermentation media. *Molecules,* **2019**, *24*(*22*), 4146. DOI: 10.3390/molecules24224146. (SBE: 90%; IF: 3,267)

P III Diána Weiser, Zoltán Boros, József Nagy, Gábor Hornyánszky, **Evelin Bell**, Péter Sátorhelyi, László Poppe: Chapter 15: SynBiocat: Protein purification, immobilization and continuous-flow processes. Biocatalysis: An Industrial Perspective, *Cambridge Royal Society of Chemistry Publishing*, **2018**, pp. 397-430. ISBN: 978-1-78262-619-0

P IV Flóra Nagy, Gábor Tasnádi, Diána Weiser, **Evelin Bell**, Mélanie Hall, Klaus Ditrich, Kurt Faber, László Poppe: Smart nanoparticles for selective immobilization of acid phosphatases. *ChemCatChem,* **2018** *53* pp. 1-11. DOI: 10.1002/cctc.201800405. (SBE: 15%; IF: 4,495)

P V Rózsa Szűcs, Diána Balogh-Weiser, **Evelin Sánta-Bell**, Eszter Tóth-Szeles, Tamás Varga, Zoltán Kónya, László Poppe, István Lagzi: Green synthesis and in-situ immobilization of gold nanoparticles and their application for the reduction of *p*-nitrophenol in continuous-flow mode. *RSC Advances,* **2019**, *9,* pp. 9193-9197. DOI: 10.1039/c8ra10373a. (SBE 100%, IF: 3,119)

P VI **Evelin Bell**, Zoltán Boros, Gábor Hornyánszky: Novel Synthesis of 1-substituted *meso*-3,4-dihydroxypyrrolidines with a $RuCl_3$-catalyzed Key Reaction. *Periodica Polytechnica-Chemical Engineering*, **2015** *59:(2)* pp. 124-128. DOI: 10.3311/PPch.7495. (SBE: 95%; IF: 0,460)

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Sánta-Bell E., Kovács N. K., Molnár Zs., Sátorhelyi P., Balogh-Weiser D., Hornyánszky G., Poppe L.: Szelektív enzimrögzítési módszerek fejlesztése mágneses nanorészecskéken, *Fiatal Biotechnológusok Országos Konferenciája*, 26 November **2020**, Hungary, Poster Prize awarded.

E. Sánta-Bell, N. K. Kovács, Z. Molnár, P. Sátorhelyi, D. Balogh-Weiser, G. Hornyánszky, L. Poppe.: Selective enzyme immobilization techniques on magnetic nanoparticles, *14th International Symposium on Biocatalysis and Biotransformation*, 7-11 July **2019**, Gröningen, Netherlands.

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