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General protein purification and immobilization method on controlled porosity glass: Biocatalytic applications

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A general combined purification and immobilization method to facilitate biocatalytic process development is presented. The support material, EziG™, is based on controlled porosity glass (CPG) or polymer-coated versions thereof (HybCPG) and binds protein affinity tags. Biocatalytic reactions in aqueous and organic media with seven enzymes of biocatalytic interest are shown.

The benefits of immobilizing an enzyme on a carrier before its use in biocatalytic reactions include increased reusability, stabilization and facile separation from the reaction mixture.[1] The immobilization of an enzyme in a biocatalytic reaction is also a requirement for high process efficacy.[2-4]

Various enzyme immobilization techniques have been published. Porous materials are often used as enzyme carriers[4] for biocatalytic reactions in organic solvent, e.g. macroporous polypropylene polymer beads (Accurel®),[5] and polymeth-acrylate.[2] Furthermore, enzymes can be coupled to one another to form cross-linked enzyme aggregates (CLEAs), consisting of almost only enzyme molecules, that can be used as a heterogeneous catalyst.[2, 6] The existing enzyme immobilization techniques are effective but not general. Testing and optimization of a number of methods are usually required before a suitable preparation is found, and pre-purification is necessary to achieve a pure enzyme catalyst. A general method that allows purification and immobilization of enzymes in one step, providing biocatalysts for organic transformations, is highly desirable.

Glass, a material inert in nature, can be produced as particles of size-controlled macro- or meso-pores, CPG, and as a hybrid inorganic-organic material thereof, HybCPG.[7, 8] These materials are suited for various applications in fluid processes, since they are stable in most organic solvents and aqueous environments at a pH below 10. The nature of the material on which an enzyme is immobilized is important for process efficacy and reusability.[9, 10] CPG is a suitable material for this purpose.[10, 11] Its average pore diameter can be varied from 10 to 300 nm with a maintained sharp pore size distribution. This provides a favorable micro-environment for enzymes, where the interconnecting pore structure facilitates the mass transfer of reactants and products throughout the material. Enzymes and other proteins have been immobilized into CPG[10, 12-15] using glutaraldehyde as a linker to aminated CPG. This linker was also used to bind enzymes to aminopropyl-mesocellular foam.[16, 17] The recently developed HybCPG[8] may offer additional benefits as an immobilized enzyme carrier, but has earlier not been explored for this purpose. In HybCPG the otherwise hydrophilic pore surface is coated with an organic polymer, e.g. homogeneous polystyrene for a hydrophobic surface, homogeneous polyacrylonitrile for a hydrophilic surface, or co-polymeric design for blended characteristics (see Supplementary Information for more details).

The aim of the present study was to construct a general method for purification and immobilization of enzymes for biotransformations based on modified CPGs and HybCPGs, which can bind protein affinity tags, named EziG™. The method was explored with seven enzymes: one ω-transaminase, three lipases, and a Baeyer-Villiger monooxygenase (BVMO) together with two cofactor-regenerating enzymes.

Three types of EziG™ were produced by the use of one type of long chain aminoalkyl CPG (LCAA CPG), a material commonly used for solid phase oligonucleotide synthesis,[18] and two types of amino HybCPG. The HybCPG types employed were custom-made products; one coated with a hydrophobic polymer including vinylbenzyl chloride units (HybCPG VBC) and another with a hydrophilic coating consisting of a copolymer of styrene and acrylonitrile (HybCPG copo). In these tests a pore diameter of 50 to 60 nm was employed.

The amino CPGs were treated with 2,4-dihydroxy-acetophenone, and the imines formed were reduced followed by Co(II) complexation (see Scheme S1, Supplementary Information). The EziG™ could thereafter directly be used as a binding matrix for His_6-tagged enzyme (Figure 1), the HybCPG-based preparations showed higher catalytic activity. Immobilization was performed with cell lysate in the case of intracellular expression or cell culture supernatant for extracellular expression, yielding a pure heterogeneous enzyme catalyst. Extraction of purified enzyme could be performed by

standard immobilized metal affinity chromatography (IMAC)-technique.

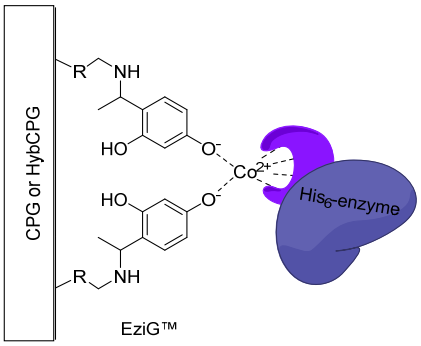
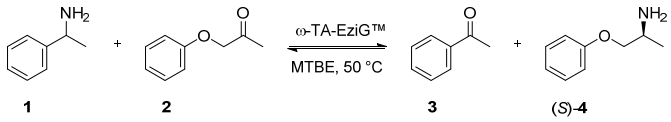


Figure 1: EziG™ with chelated Co(II) and bound His_6-enzyme. The structure of the R-group is not revealed, and varies between the CPG products.

Chromobacterium violaceum ω-transaminase (ω-TA)[19-21] was immobilized on EziG™ by immersing the carrier material in cell lysate with overexpressed enzyme; binding was completed within 20 min on an orbital shaker. The yield of immobilization was measured by active site quantification[22] to ensure that deactivated enzyme was not included in the measurement, by measuring the active sites in the cell lysate before and after immobilization and comparing that difference with the active sites measured on the obtained ω-TA-EziG™. More than 99% of the extracted enzyme was active; the enzyme mass corresponding to the found active sites on the carriers yielded an enzyme mass loading of more than 20%. ω-TA-EziG™ was successfully used as transamination catalyst in a model reaction in methyl *tert*-butyl ether (MTBE), (see Scheme 1) in which lyophilized cell lysate has previously been reported to be active;[23] see Table 1 and Table S1 (Supplementary Information). In this model reaction transamination of 1-phenoxypropan-2-one (**2**) with 1-phenylethylamine (**1**) afforded enantiomerically pure (*S*)-1-phenoxypropan-2-amine ((*S*)-**4**, >99% ee) and acetophenone (**3**). The lyophilized cell lysate from our preparation did not display any catalytic activity under the tested reaction conditions.



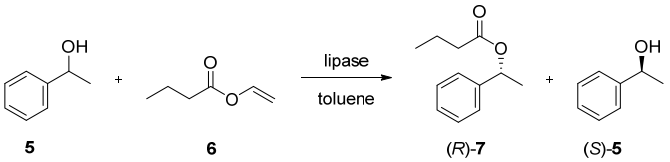
Scheme 1: Chosen model reaction for ω-TA-EziG™, where 1-phenylethylamine **1** (150 mM) was used as amino donor in the transamination of 1-phenoxypropan-2-one **2** (50 mM) in methyl *tert*-butyl ether (MTBE).

Table 1: Immobilization yield, enzyme loading, enantiomeric excess of the product (ee_p) and initial rate for ω-TA-EziG™ prepared from different types of CPG, for the reaction depicted in Scheme 1.

CPG type	Yield of immobilization (% active enzyme)	Active enzyme loading (% w/w)	ee_p (%, GC)	Initial rate (μmol/min/g EziG™)
LCAA CPG	>99%	24	>99 (*S*)	0.38
HybCPG VBC	>99%	29	>99 (*S*)	0.70
HybCPG copo	>99%	21	>99 (*S*)	0.06

ω-TA-EziG™(HybCPG VBC) was the most active preparation under the conditions tested, plausibly due to the hydrophobic nature of the surface of the carrier, which may prevent denaturation. However, the reason for the observed differences in reaction rate with the tested carriers remains to be fully investigated. Our results demonstrate that the enzyme functions in MTBE in a pure form on a carrier with a measureable active enzyme amount.

Candida antarctica lipase B (CalB)[24, 25] was immobilized on EziG™ from cell culture supernatant after expression and secretion by *Pichia pastoris*. For comparison immobilization was also done on Accurel®, where 8 h of incubation was required. EziG™ immobilization was achieved within 30 min. The immobilized CalB preparations CalB-EziG™ and CalB-Accurel® were used in kinetic resolution of 1-phenylethanol (**5**) with vinylbutyrate (**6**) as acyl donor (Scheme 2). The enantiomeric excess (ee) of (*R*)-1-phenylethyl butyrate ((*R*)-**7**) was determined by chiral GC. The ee, enantioselectivity (*E*), and the activities are given in Table 2. An excellent enantioselectivity (E > 300) was obtained for both CalB-EziG™ and CalB-Accurel®.



Scheme 2: Model reaction for immobilized lipases; the kinetic resolution of 1-phenylethylamine **5** (10 mM) by the transesterification with vinylbutyrate **6** (100 mM).

Table 2: Incubation time required for immobilization, *E* value, ee, and bulk volumetric activity for a chosen model reaction (Scheme 2, 22 °C), for four preparations of immobilized CalB.

Carrier[a]	Incubation time for immobilization (h)	*E*	ee of (*R*)-**7**[b] (%, GC)	Initial rate per bulk volume[c] (μmol/min/cm^3 carrier)
EziG™ (LCAA CPG)	0.5	>300 (*R*)	>99.5	0.23
EziG™ (HybCPG VBC)	0.5	>300 (*R*)	>99.5	0.21
EziG™ (HybCPG copo)	0.5	>300 (*R*)	>99.5	0.26
Accurel®	8	>300 (*R*)	>99.5	<0.60[d]

[a] See Table S1 (Supplementary Information) for EziG™ data. [b] Calculated at 49% conversion. [c] Initial rate of consumption of **5**. [d] Without inclusion of swelling which occurs in contact with solvent.

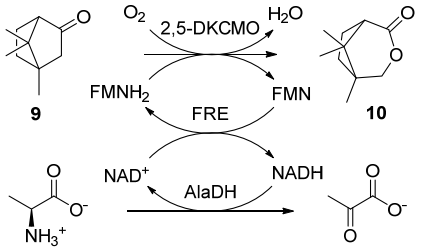
The activity for CalB-Accurel® is approximately twice that of CalB-EziG™ per bulk volumetric unit (volume occupied by the catalyst including the pore volume) when calculated on the dry preparations, as shown in Table 2, and approximately six times higher per mass unit since the EziG™ is a more dense material, see Table S2 (Supplementary Information). In the reaction mixture the Accurel® swells to approximately double the volume while the EziG™ is virtually unchanged, therefore the catalysts have approximately equal catalytic activity per bulk volume *in situ* (data not shown).

The CalB variant Trp104Ala has previously been shown to have higher activity and reversed enantiopreference for bulky secondary alcohols compared to the wild-type enzyme.[26-28] This enzyme was readily bound to EziG™ within 30 min of incubation while ten days of incubation was required for immobilization on Accurel®. For this variant, EziG™ (HybCPG VBC) is a more suitable carrier than Accurel® based on the bulk volumetric activity and the time needed for immobilization (see Table S2, Supplementary Information).

Candida antarctica lipase A (CalA),[29-31] a lipase with a wider substrate range and lower enantiospecificity compared to CalB, was immobilized on EziG™ (LCAA CPG) and applied

for the chosen model reaction (Scheme 2). As expected the *E* value was low (1.3), but with a higher activity than that of CalB (Table S2, Supplementary Information). The results show that EziG™ is suitable for immobilization of CalA and further optimizations with subsequent applications are ongoing.

The $FMNH_2$-dependent BVMO 2,5-diketocamphane monooxygenase from *Pseudomonas putida* (2,5-DKCMO)[32] was co-immobilized on EziG™ with two cofactor-reconverting enzymes (flavin reductase (FRE) from *E. coli*[33, 34] for $FMNH_2$ recycling and alanine dehydrogenase (AlaDH) from *B. subtilis*[35] for NADH recycling). This immobilization was performed by exposing the EziG™ carrier to a mixture of all three cell lysates after overexpression of the three enzymes. The three-enzyme EziG™ was then used for an enzymatic cascade reaction for Baeyer-Villiger oxygenation of (+)-camphor (**9**) (2.0 mM) in phosphate buffer, with $FMNH_2$ and NADH regeneration by consumption of L-alanine (Scheme 3). The reaction was started by addition of molecular oxygen followed by sealing of the reaction vessel and subsequent orbital shaking. A preparation with EziG™ based on LCAA CPG displayed virtually no conversion, whereas the HybCPG VBC preparation gave 63% of lactone **10** within 3 h. The latter vessel was left for 24 h without any improvement of conversion; however, re-addition of O_2 and reaction for another 3 h increased the yield of **10** to 88%. An analogous experiment with dissolved enzymes showed conversion but could not be restarted by addition of O_2, presumably due to deactivation of the unstable 2,5-DKCMO. These results show that EziG™ immobilization had a stabilizing effect on one or several of the enzymes, and is effective for the co-immobilization of several His_6-tagged enzymes from the same mixture for cascade reactions.



Scheme 3: Enzymatic cascade reaction with EziG™ co-immobilized single-component BVMO (2,5-DKCMO), FRE and AlaDH for oxidizing (+)-camphor **9** to 5,8,8-trimethyl-3-oxabicyclo[3.2.1]octan-2-one **10**, L-alanine conversion to pyruvate is used for recycling the cofactors NAD^+ and $FMNH_2$.

In a preparative experiment a multi-phase reaction system was used for the oxidation of (+)-camphor (**9**) to lactone **10** using **9** in cyclohexane (100 mM) together with the aqueous reaction mixture described above (Figure S1, Supplementary Information). The EziG™-preparation remained in the aqueous phase while mild orbital shaking was applied due to its density. The immobilized enzymes were thereby protected from deactivation through contact with the solvent interface. The three-phase (cyclohexane, aqueous phosphate buffer, and solid EziG™) reaction system showed 56% conversion to lactone **10** after 72 h. The analogous experiment with dissolved enzymes in a two-phase environment gave virtually no conversion within the same time period.

Schulz *et. al.* have reported a successful enzymatic cascade BVMO-reaction with a single-component BVMO,[36] which does not require a distinct flavin reductase for its supply of $FADH_2$. The method described herein for co-immobilization of a three-enzyme-based cascade reaction system opens up new possibilities for applying the more demanding two-component BVMOs in biocatalytic transformations.

Conclusions

EziG™ was used for combined protein purification and immobilization of seven enzymes from cell lysate or cell culture supernatant, which were then employed in biocatalytic reactions. ω-TA-EziG™ was active in MTBE. CalA- and CalB-EziG™ were active transesterification catalysts and could be obtained in a significantly shorter time compared to the corresponding Accurel®-based preparations. Co-immobilization of 2,5-DKCMO, FRE and AlaDH on the same EziG™-carrier led to increased stability in a two-phase system and resulted in a functioning enzymatic cascade reaction. EziG™ based on HybCPG was shown to be the most suitable carrier for all enzymes tested; HybCPG enables tailoring of the surface on which the enzyme is bound.

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Notes and references

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Electronic Supplementary Information (ESI) available: [additional information regarding the enzymes used in this study, the carrier material and the biocatalytic example reactions, and materials and methods]. See DOI: 10.1039/c000000x/

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