Bio-catalytic cascades and molecular oxygen-accessing amines and nitriles

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Chapter 3

Reductive amination using agarose-entrapped amine dehydrogenase in a continuous flow reactor

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‡ These authors equally contributed to this work.
Chapter 3

3.1 Introduction

In recent years, flow chemistry has proven to be an important technology both in academic and industrial settings.\textsuperscript{1,2} For instance, its potential was highlighted among the emerging technologies by the IUPAC to aid ongoing quest towards more sustainable industry.\textsuperscript{3} Simultaneously, biocatalysis is regarded as one among the most impactful green synthetic disciplines as it enables for instance mild and selective approaches to afford fine chemicals.\textsuperscript{4} An excellent example of industrial biocatalytic process is the synthesis of Islatravir that was developed through a collaboration between Merck and Codexis.\textsuperscript{5} Therefore, it is understandable that the combination of these two technologies attracted significant attention and holds a great potential.\textsuperscript{1,6,7} In this context, enzyme immobilization techniques play a pivotal role in “flow-biocatalysis”, since they allow us to localize the catalysts in a specific compartment of the flow apparatus while substrates and reagents stream through.\textsuperscript{7,8} Additionally, immobilization

![Figure 3.1](image-url)

Figure 3.1. In-flow reductive amination using co-immobilized LE-AmDH-v1 and CbFDH. a) General scheme of the flow-system b) Reaction scheme. BPR, back-pressure regulator.
of biocatalyst brings along major additional benefits such as the decrease of the required quantity of enzyme, simplification of product work-up and potential increase of enzyme stability. Recently, flow-based approaches have found their way into the biocatalytic synthesis of amines. There have been several remarkable applications utilizing ω-transaminases in flow, among which contributions of our group were achieved using EziG immobilization support. In general, EziG beads are controlled porosity glass (CPG) material coated with a polymeric surface of diverse hydrophobic or hydrophilic nature and bearing chelating groups, which are suitable for selective binding of metal ions (Fe$^{3+}$). A subsequent chelation between these cations and the poly-histidine tag—recombinantly fused to the target protein—occurs upon contacting enzyme and carrier in solution. Since we, and independently also Turner’s group, have previously demonstrated the compatibility between EziG beads and amine dehydrogenases, it was clear that we should harness the catalytic properties of our recently engineered amine dehydrogenase (LE-AmDH-v1) and test it in a flow-based process (Figure 3.1a). More specifically, we have decided to investigate the synthetic applicability of LE-AmDH-v1, co-immobilized with formate dehydrogenase from Candida boiindii (CbFDH), on EziG carrier (Figure 3.1b – reaction scheme) by performing the reductive amination of benzaldehyde (1a) to benzylicamine (1b).

## 3.2 Results and discussion

### 3.2.1 Co-immobilization of LE-AmDH-v1 and CbFDH

The first attempt to co-immobilize LE-AmDH-v1 and CbFDH was performed using EziG-Amber metal-ion affinity beads. Aiming for the simplest possible methodology, we initially tested co-immobilization of LE-AmDH-v1 and CbFDH since the selectivity of the ion-affinity binding enables immobilization directly from the cell lysate, thus effectively precluding the need for enzyme pre-purification. Initial batch reactions afforded low levels of analytical yields of 1b (Figure 3.2). However, we were satisfied with the fact that the enzymes retained their activity. Subsequently, we attempted to perform the reductive amination directly in flow using co-immobilized LE-AmDH-v1 and CbFDH. However, we observed adsorption of the substrate onto the EziG-Amber beads.
because we could not recover the full amount of the unreacted substrate in the flow-through fractions. Moreover, we observed subsequent elution of the adsorbed benzaldehyde long after the elution of the reaction volume from the column. Interestingly, this problem occurred only when 1a was used as a substrate. For example, acetophenone did not get retained by the Amber beads at all, proving the incompatibility of the aldehyde reagent with this type of immobilization support. Since we still wanted to test the amination of the more challenging substrate 1a—due to its higher chemical reactivity and volatility that complicate batch reactions—the substrate-carrier incompatibility prompted us to investigate alternative immobilization materials and methodologies. For instance, we tested EziG-Opal beads and Purolite resin (the latter containing pre-loaded with Co²⁺ ions) using again His-tag affinity binding. Another option was the use of three support materials that rely on the covalent attachment of the protein to the material through spatially random epoxide-amine linkage (Sepabeads EC-EP/s and Relizyme 113/s and 403/s). In order to better monitor the progress of immobilization, we used purified enzyme in this experiment (LE-AmDH-v1/CbFDH, molar ratio of 90:16), as reported previously.¹⁷ Moreover, we also monitored the
immobilization using SDS-PAGE by comparing the electrophoresis traces of samples taken before and after immobilization procedure. We observed the best conversions using the EziG-Opal and Purolite (Co²⁺) resin with the enzymes (>96% of analytical yield of 1b), whereas none of the enzymes covalently bound to any support could afford analytical yields higher than 20% (Figure 3.3a). Moreover, the immobilization of LE-AmDH-v1/CbFDH onto EziG-Opal and Purolite resin exhibited 50% and 80% immobilization efficiency, respectively (Figure 3.3b). Ultimately, we have decided to continue our study using EziG-Opal due to their superior handling and apparent possibility to be utilized for in-flow immobilization. Next, we tested the efficiency of the immobilization using the EziG-Opal over the period of four hours using 5%, 10% or 15% w w⁻¹ loading of the biocatalysts. The analysis revealed almost full immobilization after 2 h in all of the conditions. Additionally, we have attempted to co-immobilize LE-AmDH-v1 and CbFDH on EziG-Opal beads using the cell lysate. The immobilization was performed by using 100 mg of beads and, with the aim of immobilizing around 10 mg of enzymes (10% w⁻¹), 0.57 g of E. coli cells containing overexpressed LE-AmDH-v1 (ca. 8.55 mg of enzyme) and 0.15 g of E. coli cells containing overexpressed CbFDH (ca. 1.45 mg of enzyme). The process was monitored via SDS-PAGE gels, resulting in an immobilization efficiency of 70-80%. Subsequently, beads were used directly for activity testing (in the usual reaction conditions) and almost full conversion of starting material (benzaldehyde) was obtained and ultimately resulting in 55% analytical yield of 1b. At this point, we cannot fully explain the discrepancy between the analytical yield and the conversion. We can speculate that a portion of the 1a could have been adsorbed onto the unspecifically bound proteins originating from the E. coli extract, since the polyhistidine-tag is suitable for binding relatively large quantities of protein, albeit it suffers from a lower specificity. The results involving EziG-Opal were looking promising at this point and therefore, we directly attempted to co-immobilize the enzymes from cell lysate in-flow. A stainless-steel column was filled with EziG-Opal materials (ca. 500 mg) and the cell lysate containing LE-AmDH-v1 and CbFDH (50 mg enzymes, 10 % w⁻¹ loading) was streamed through the column using a peristaltic pump. SDS-PAGE gel analysis showed that almost full immobilization was obtained. Afterwards the column was mounted on a HPLC pump,
and the beads inside the column were conditioned by flowing reaction buffer (ammonium formate 750 mM, pH 8). However, we observed a change in color of the

Figure 3.3. Co-immobilization of LE-AmDH-v1 and CbFDH on different immobilization supports: a) activity testing; b) investigation of immobilization efficiency via SDS-PAGE.
buffer exiting the column during this conditioning step. Unfortunately, SDS-Page analysis confirmed our suspicion that the beads did not retain the enzymes in presence of the reaction buffer (Figure 3.4a). We attempted to overcome this issue of leaching of the enzymes from the column by introducing a further cross-linking immobilization step on the EziG-enzyme system using glutaraldehyde (5% v v⁻¹ solution). Therefore, the glutaraldehyde solution in KPi buffer (100 mM, 7.6 pH) was quickly streamed through the column right after that all the cell lysate had been loaded inside, and then a washing step was carried out with Tris-HCl (pH 8, 100 mM). The column was then used for the in-flow biotransformation. Although we did not observe any leaching of the enzymes (Figure 3.4b), the catalyst inside the column apparently lost its catalytic activity since no product was detected. Therefore, it appears that EziG-Opal is not suited for the immobilization in the environment containing large excess of ammonium ions due to enzyme leaching. Additionally, EziG-Amber appears to be unsuitable for the reductive amination of benzaldehyde due to excessive adsorption of the substrate onto the support.
3.2.2 Co-entrapment of LE-AmDH-v1 and Cb-FDH on agarose-based hydrogel

The many issues experienced for the co-immobilization of LE-AmDH-v1 and CbFDH spurred us to search for more synthetically viable solutions. We turned our attention towards the entrapment of the enzymes inside hydrogels, since encapsulation of enzymes in natural and synthetic polymers in form of hydrogels represents a milder procedure for physical entrapment without the need for enzyme-specific adaptations.19 More specifically, we were particularly inspired by the pioneering work of Rabe’s group. They have reported the use of 3D-printed agarose gel reactors19,20 for the flow-biocatalysis. The process itself involves addition of the target enzyme into the molten agarose and subsequent printing of the custom bio-reactor using a 3D-printer. Moreover, since the printing process is performed at 60°C in order to keep the agarose liquid, we could harness the thermostability of LE-AmDH-v117 (Tm over 60 °C). Unfortunately, several issues were encountered from the start with the 3D printing process of the reactor as reported in the original publication, probably due to different features of the 3D printer used in our laboratory. In particular, the necessity to use a 3D-printer equipped with a heated tip along with a heated agarose-container was the reason for this discrepancy as our device does not have the heated tip feature. We decided to circumvent this limitation by designing and 3D-print a mold and an insert (Figure 3.5), in order to cast the reactor rather than to print it. Moreover, the avoidance

Figure 3.5. Different types of molds and agarose-based reactors: a) short design with multiple (15) small pores; b) long design with small number of pores (4).
of the printing step could broaden the applicability of “agarose-based reactor system” to the biocatalysts originating from mesophilic organisms. In fact, the 3D-printing required 60 °C for the agarose to remain liquid in the 3D-printer, whereas casting the reactor allows us to use the agarose at 50 °C. Initial activity tests involving LE-AmDH-v1 and CbFDH entrapped in agarose-based reactor were performed in batch. In order to prepare the reactors in a reproducible fashion, agarose was molded as cylindrical gel ‘bricks’ with a dimension of ca. 0.5 cm x 0.5 cm (diameter x height), containing LE-AmDH-v1 and CbFDH in 90 μM and 16 μM as final concentration (ca. 1 mL of agarose solution). The resulting bricks were placed into 2 ml Eppendorf tubes and they were directly tested in biotransformations using ammonium formate buffer (750 mM, pH 8) and 1a (10 mM). The results seemed promising, since we observed quantitative conversion of the starting material together with 90% analytical yield of benzylamine product. Interestingly, an aliquot of the product remained initially adsorbed in the hydrogel reactor, since we obtained ca. 70% analytical yield of 1b when the agarose

Figure 3.6. Recycling of the agarose-based reactor containing LE-AmDH-v1 and CbFDH in batch. Reactor age is given at the beginning of the experiment. Reaction conditions: ammonium formate (750 mM, pH 8, supplemented with NAD+ 1 mM), 1a (10 mM), 1 cm x 1 cm gel brick with LE-AmDH-v1 (90 μM) and CbFDH (16 μM) incubated for 24 h, at 40 °C and 170 rpm.
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reactor was not subjected itself to an extraction step. Next, we decided to investigate the retention of the catalytic activity of entrapped enzymes in agarose-based hydrogel bricks over time. Thus, we performed the reaction over the course of 9 days (Figure 3.6). The reaction revealed that the catalytic activity was well retained until the 3rd cycle and dropped down in the 4th cycle (equal to 6 days reactor operation). The highest activity was observed in the 1st and 2nd cycles, with analytical yields of 1b ranging between 80-100%. With the functional casting protocol in hand, we decided to apply the agarose-based hydrogel with entrapped LE-AmDH-v1 and CbFDH in a flow system. We adapted the 3D-printing of the mold in order to prepare a long agarose-based reactor (1 cm x 6 cm, diameter x length) that could fit directly into an empty HPLC column and perform the reductive amination in flow (Figure 3.1). The process afforded quantitative conversion and >95% analytical yield of 1b, which suggests that our approach is suitable for running such reactions.
3.3 Conclusion

Flow-biocatalysis appears to be an attractive alternative to traditional batch biotransformations. In this work, we studied our novel engineered amine dehydrogenase LE-AmDH-v1 co-immobilized with CbFDH onto supports belonging to the commercial EziG and Purolite platforms. However, we discovered that the EziG-Amber carrier causes adsorption of benzaldehyde on the surface of the beads, whereas the EziG-Opal releases a significant amount of co-immobilized enzymes upon contact with ammonium formate buffer (750 mM, pH 8) required for the reductive amination reaction. Failing to apply the EziG platform due to the process specific limitations, we investigated the applicability of the 3D-print agarose-gel reactors containing entrapped LE-AmDH-v1 and CbFDH. However, we could not fully evaluate that due to hardware issues associated with the 3D-printing process and our available 3D-print device. Conversely, we devised an alternative approach entailing the cast of an agarose-based reactor containing entrapped LE-AmDH-v1 and CbFDH. We investigated the possibility to use the reactor for reductive amination over the course of several days and ultimately, we performed reductive amination of benzaldehyde over the course of five days with 80% isolated yield. We stand convinced that the flow-based reductive amination using casted agarose reactors is a beneficial addition to the available biocatalytic methodologies, especially due to the versatility enabled by 3D printing.
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3.4 Experimental

3.4.1 Initial investigation on co-immobilization of LE-AmDH-v1 and CbFDH

3.4.1.1 Expression and purification of LE-AmDH-v1 and CbFDH

Recombinant expression and subsequent purification of LE-AmDH-v1 and CbFDH was performed as reported in Tseliou et. al.\textsuperscript{17} Moreover, the procedure for purification of His-tagged proteins can be found in Chapter 6 (section 6.4.1.3).

3.4.1.2 Co-immobilization using cell free extract of LE-AmDH-v1 and CbFDH onto EziG-Amber beads

The bacterial cell pellet containing overexpressed LE-AmDH-v1 or CbFDH was thawed and re-suspended in 0.9% Tris-HCl (100 mM, pH 7.8, 1 g of cells in 5 ml of buffer) and subsequently sonicated (5 min, 10 s on, 10 s off, 45% amp). Afterwards, samples were centrifuged (10 min, 4 °C, 14800 rpm) and the supernatant was filtered (0.45 μm filter pores) and collected.

Co-immobilization of LE-AmDH-v1 and CbFDH on EziG metal-ion affinity beads was carried on as follows. To a 15 ml falcon tube containing 200 mg (20% loading w w\textsuperscript{-1}) of EziG-Amber carrier material, crude cell-free extract (i.e., CFE) containing a mixture of overexpressed LE-AmDH-v1 and CbFDH was added. The mixture was shaken with an orbital shaker (120 rpm) for 3 hours at 4 °C. Small aliquots from the aqueous phase (20 μl) were sampled before and after the immobilization procedure and the progress of the immobilization was monitored through SDS-PAGE. The immobilized enzyme was left to sediment, the buffer was removed by pipetting, and the immobilized enzyme was used directly in biotransformations.

Reaction conditions: 1 ml final volume in 2 ml Eppendorf tubes; Ammonium formate buffer (375 and 750 mM, pH 8); NAD\textsuperscript{+} (1mM); 1a (10 and 20 mM) as substrate. Co-immobilized LE-AmDH-v1 and CbFDH were prepared as reported above (ca. 25 mg each sample). The biotransformations were incubated at 170 rpm and 30 °C in an orbital shaker for 24 h. After reaction time was up, 500 μl of the reaction mixture was
quenched with 70 µl of 10 M KOH, extracted once with 650 µl of EtOAc containing 10 mM toluene as internal standard (1 min vortexing + 5 min centrifugation, 4 °C, 14800 rpm) and dried over MgSO₄. Analytical yields were determined by GC-FID (see paragraph Analytics) using toluene as internal standard. Reactions were performed in duplicate.

Table 3.1. Reductive amination of benzaldehyde (1a) using immobilized LE-AmDH-v1. Reaction conditions: ammonium formate (375 and 750 mM, pH 8, supplemented with NAD⁺ 1 mM), 1a (10 and 20 mM), co-immobilized LE-AmDH-v1 and CbFDH on EziG-Amber beads (ca. 25 mg each sample), 22 h, 30 °C, 170 rpm.

<table>
<thead>
<tr>
<th>Substrate Loading [mM]</th>
<th>Buffer Concentration [mM]</th>
<th>Analytical Yield of 1b (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>375</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>20</td>
<td>375</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>10</td>
<td>750</td>
<td>38 ± 5</td>
</tr>
<tr>
<td>20</td>
<td>750</td>
<td>43 ± 7</td>
</tr>
</tbody>
</table>

3.4.1.3 Co-immobilization in flow using cell free extract of LE-AmDH-v1 and CbFDH onto EziG-Amber beads

Cell pellet was obtained as described in previous section. A stainless-steel column (50 mm length x 2 mm diameter) was filled with EziG-Amber beads (500 mg) and hydrated with Tris-HCl buffer (50 ml, 100 mM, pH 7.8, flow 0.5 ml min⁻¹). The soluble protein fraction of CFE prepared as reported in section 4.1.3 (ca. 100 mg total enzymes, 20 % w w⁻¹) was loaded onto the column using a peristaltic pump (flow rate = 150 µl min⁻¹). After complete loading, the flow was stopped, and the cell lysate was left to incubate in the column for 45 min at room temperature. Then, Tris-HCl buffer (50 ml, 100 mM, pH 7.8, flow 0.5 ml min⁻¹) was flowed through the column to wash out any possibly unspecifically and weakly bound component. Buffer samples (20 µl) of the loading enzyme solution and of the flow-through obtained during washing step were taken and immobilization was assayed through SDS-PAGE.

The column containing the co-immobilized enzymes was subsequently mounted on a Dionex P680 HPLC pump unit and conditioned by flowing further with ammonium formate buffer (30 ml, 750 mM, pH 8, flow 0.3 ml min⁻¹). Then, reaction mixture was
injected into the system directly from the HPLC solvent lines at a flow of 1 ml min$^{-1}$. When all of the solution was injected, HPLC line was switched to reaction buffer containing reservoir. Then, flow rate was kept constant until all of the void volume was flashed out (ca. 13 ml). Subsequently, HPLC output was connected to the column containing co-immobilized enzymes and the flow rate was set at 0.01 ml min$^{-1}$. Column was warmed up at 50 °C using a heated water bath, and the system was maintained at constant pressure through a BPR element (40 psi). The reactor outcome was collected in 2 ml Eppendorf tubes (ca. 1 ml in each tube) and the reaction was stopped with 70 μl of 10 M KOH, extracted once with 650 μl of EtOAc containing 10 mM toluene as internal standard (1 min vortexing + 5 min centrifugation, 4 °C, 14800 rpm) and dried over MgSO$_4$. Analytical yields were determined by GC-FID (see paragraph Analytics) using toluene as internal standard.

Reaction mixture: 5 ml final volume; 1a (20 mM) in ammonium formate buffer (750 mM, pH 8); NAD$^+$ (1 mM).

Reaction afforded 31.7% of analytical yield of 1b.

3.4.2 Co-immobilization on alternative carriers

3.4.2.1 EziG-Opal

A 15 ml Falcon tube containing 100 mg of EziG-Opal carrier material was cooled down in an ice bath and suspended in the immobilization buffer (Tris-HCl, 10 ml, 100 mM, pH 7.8). Purified LE-AmDH-v1 and CbFDH (10 mg, equal to 10 % w w$^{-1}$, enzyme loading to support material) were added to the suspension (335 μl of LE-AmDH-v1 from a 572 μM stock solution; 26 μl of CbFDH from a 1.33 mM stock solution), and the mixture was shaken with an orbital shaker (120 rpm) for 3 hours at 4 °C. Small aliquots from the aqueous phase (30 μl) were sampled before and after the immobilization procedure, their concentrations were determined using Bradford and monitored with SDS-PAGE. The immobilized enzyme was left to sediment, the buffer was removed by pipetting, and the immobilized enzyme was used directly in biotransformations.
3.4.2.2 Purolite resin

Purolite resin loaded with Co²⁺ (300 mg) was washed with KPi buffer (2 x 0.6 ml, 100 mM, pH 7.5). Buffer was removed after 5 minutes of manual shaking. Short pulse centrifugation was used to sediment the resin and the buffer was removed by pipetting. Purified LE-AmDH-v1 and CbFDH (10 mg; 335 μl of LE-AmDH-v1 from a 572 μM stock solution; 26 μl of CbFDH from a 1.33 mM stock solution) were prepared in KPi buffer (100 mM, pH 7.5) and added to the resins (1.2 ml final volume, each). The vials (2 ml) were shaken for 2 h at 4 °C using an orbital shaker (90 rpm, horizontal positioning of vials). Small aliquots from the aqueous phase (30 μl) were sampled before and after the immobilization procedure, their concentrations were determined using Bradford and monitored with SDS-PAGE. Immobilized enzyme was washed with KPi buffer (2 x 0.6 ml) and then incubated for 45 min in KPi buffer (1 x 1.2 ml). Finally, another washing step was performed with KPi buffer (2 x 0.6 ml) and the immobilized enzyme was ready for activity testing.

3.4.2.3 Various co-valent resins

Epoxy-functionalized Sepabeads EC-EP/S, Relizyme 113/S and 403/S (100 mg each) were suspended in 5 ml KPi (100 mM, pH 7.5) in three different tubes and purified LE-AmDH-v1 and CbFDH (10 mg; 335 μl of LE-AmDH-v1 from a 572 μM stock solution; 26 μl of CbFDH from a 1.33 mM stock solution) were added in each tube. The mixture was shaken on an orbital shaker at 4 °C for 72 hours (90 rpm, mild shaking). After centrifugation (10.0 krpm, 1 min, 4 °C) buffer solution was discarded and the beads were washed with KPi (100 mM, pH 7.5, 1 ml) for several minutes (600 rpm). After centrifugation, again the remaining solution was discarded and the beads were washed with KPi (100 mM, pH 7.5, 1 ml) for 30 min (600 rpm). After centrifugation, the supernatant was discarded and beads were directly used in the activity measurements. Small aliquots from the aqueous phase (30 μl) were sampled before and after the immobilization procedure and during the washing steps, their concentrations were determined using Bradford and monitored with SDS-PAGE. Reaction conditions: 1 ml final volume in 2 ml Eppendorf tubes; ammonium formate buffer (750 mM, pH 8); NAD⁺ (1mM); 1a (10 mM) as a substrate. Co-immobilized
LE-AmDH-v1 and CbFDH prepared as reported above (ca. 25 mg for each sample). The biotransformations were incubated at 170 rpm and 30 °C in an orbital shaker for 24 h. After reaction time was up, 500 µl of the reaction mixture was stopped with 70 µl of 10 M KOH, extracted once with 650 µl of EtOAc containing 10 mM toluene as internal standard (1 min vortexing + 5 min centrifugation, 4 °C, 14800 rpm) and dried over MgSO₄. Analytical yields were determined by GC-FID (see paragraph Analytics) using toluene as internal standard. Reactions were performed in duplicate.

Table 3.2. Activity tests of co-immobilized LE-AmDH-v1 and CbFDH on different beads. Conditions: ammonium formate (750 mM, pH 8, supplemented with NAD⁺ 1 mM), 1a (10 mM), ca. 25 mg of beads, incubated for 24 h at 40 °C and 170 rpm.

<table>
<thead>
<tr>
<th>Carrier materials</th>
<th>Analytical Yield of 1b (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EziG-Opal</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>Purolite</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Sepabeads EC-EP/s</td>
<td>11 ± 0</td>
</tr>
<tr>
<td>Relyzime 113/s</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Relyzime 403/s</td>
<td>2 ± 0</td>
</tr>
</tbody>
</table>

3.4.3 Co-immobilization of LE-AmDH-v1 and CbFDH: time dependence and biocatalysts loading

10 ± 0.2 mg of EziG-Opal carrier material were suspended in the immobilization buffer (Tris-HCl, 3x1 ml, 100 mM, pH 7.8) in 3 separated vials. Purified LE-AmDH-v1 and CbFDH were added to the suspension:
-0.5 mg, equal to 5 % w w⁻¹, enzyme loading to support material; (16.8 µl of LE-AmDH-v1 from a 572 µM stock solution; 1.3 µl of CbFDH from a 1.33 mM stock solution);
-1 mg, equal to 10 % w w⁻¹, enzyme loading to support material; (34 µl of LE-AmDH-v1 from a 572 µM stock solution; 2.6 µl of CbFDH from a 1.33 mM stock solution);
-1.5 mg, equal to 15 % w w⁻¹, enzyme loading to support material; (50 μl of LE-AmDH-v1 from a 572 μM stock solution; 3.9 μl of CbFDH from a 1.33 mM stock solution).

Mixtures were shaken with an orbital shaker (120 rpm) for 4 h at 4 °C. Small aliquots from the aqueous phase (20 μl) were sampled before and after 30 min, 1 h, 2 h, 3 h and 4 h; their concentrations were determined using Bradford and monitored with SDS-PAGE.

Figure 3.6. SDS-PAGE gel of pre- and after co-immobilization of LE-AmDH-v1 and CbFDH onto EziG-Opal carrier material. Protein ladder: PageRuler™. Unstained Protein Ladder (ThermoFisher Scientific). Coomasie blue staining method.

3.4.4 Co-immobilization in flow using cell free extract of LE-AmDH-v1 and CbFDH onto EziG-Opal beads

Flow-based co-immobilization of LE-AmDH-v1 and CbFDH and subsequent activity testing were performed in the same way as in case of EziG-Amber (section 3.4.1.2). Overall result was 79.1% of residual starting material (1a); 14.3% analytical yield of 1b.
3.4.5 Cross-linking of immobilized LE-AmDH-v1 and CbFDH

Co-immobilization was performed as described in section 3.4.1.2. In order to crosslink the proteins using glutaraldehyde, the column containing the co-immobilized enzymes was subsequently mounted on a Dionex P680 HPLC pump unit and conditioned by flowing further with Ammonium formate buffer (20 ml, 750 mM, pH 8, 0.3 ml min\(^{-1}\)). Then, reaction mixture was injected into the system directly from the HPLC solvent lines at 1 ml min\(^{-1}\) and when all of the solution was injected, HPLC line was switched to reaction buffer containing reservoir. Then, flow rate was kept constant until all the dead volume was flashed out (ca. 13 ml). Subsequently, HPLC output was connected to the column containing co-immobilized enzymes and the flow rate was set at 0.02 ml min\(^{-1}\). Column was warmed up at 50 °C using a heated water bath, and the system was maintained at constant pressure through a BPR element (40 psi). The reactor outcome was collected in 2 ml Eppendorf tubes (ca. 1 ml in each tube) and the reaction was stopped with 70 μl of 10 M KOH, extracted once with 650 μl of EtOAc containing 10 mM toluene as internal standard (1 min vortexing + 5 min centrifugation, 4 °C, 14800 rpm) and dried over MgSO4. Analytical yields were determined by GC-FID (see paragraph Analytics) using toluene as internal standard.
Results – 51.6% residual 1a; 0% analytical yield of 1b.

![Figure 3.8. SDS-PAGE gel of pre- and after in-flow co-immobilization of LE-AmDH-v1 and CbFDH onto EzIG-Opal carrier material from CFE and following cross-linking. Protein ladder: PageRuler™. Unstained Protein Ladder (ThermoFisher Scientific). Coomasie blue staining method.]

3.4.6 3D-printing of molds

Molds and inserts (further addressed as devices) were designed using Autodesk Inventor (Autodesk, San Rafael, CA, USA). The devices were fabricated using stereolithography (building the object layer by layer in the desired shape via photopolymerization of liquid resin by a scanning laser or a digital light projector) using a Form 2 3D-printer (Formlabs, Somerville, Massachusetts, United States). 3D printed devices were post-processed by sonication in 2-propanol and compressed air in order to remove any uncured resin. Finally, parts were placed in a Form Cure (405 nm; Formlabs, Somerville, Massachusetts, United States) for UV and thermal curing and cured for 60 min at 60 °C.

3.4.7 Co-entrapment of LE-AmDH-v1 and CbFDH inside agarose-based hydrogel

3.4.7.1 Activity testing in batch

An agarose solution (3% w w⁻¹, supplemented with NaCl 10 mM, 2 ml final volume) was prepared in Tris-HCl buffer (100 mM, pH 7.8) and heated up with microwave (300 W,
10 sec) until a clear solution was obtained. Afterwards, the solution was allowed to cool for up to 2-3 minutes and then purified LE-AmDH-v1 (90 μM as final concentration) and CbFDH (16 μM as final concentration) were added. Quickly the resulting mixture was poured into an Eppendorf rack’s hole and allowed to cool down and solidify. Finally, the solid brick was removed from the mold, split in two equal parts and directly used for biotransformation. Reaction conditions were as follows: 1 mL final volume in 2 mL Eppendorf tubes; ammonium formate buffer (750 mM, pH 8); NAD^+ (1 mM); 1a (10 mM) as substrate. The biotransformations were incubated at 170 rpm and 40 °C in an orbital shaker for 24 h. After reaction time was up, 500 μl of the reaction mixture was stopped with 70 μl of 10 M KOH, extracted once with 650 μl of EtOAc containing 10 mM toluene as internal standard (1 min vortexing + 5 min centrifugation, 4 °C, 14800 rpm) and dried over MgSO_4_. Furthermore, the second half of the reaction mixture (500 μl) containing also the agarose-based hydrogel brick were extracted with the same procedure described above. Analytical yields were determined by GC-FID (see paragraph Analytics) using toluene as internal standard.

Figure 3.9. Hydrogel bricks inside a 2 ml Eppendorf tubes.

3.4.7.2 Preparation of agarose-based flow reactors

An agarose solution (3% w w\(^{-1}\), supplemented with NaCl 10 mM, 8 ml final volume) was prepared in Tris-HCl buffer (100 mM, pH 7.8) and heated up with microwave (300 W, 10 sec) until a clear solution was obtained. Afterwards, the solution was allowed to cool for up to 2-3 minutes and then purified LE-AmDH-v1 (90 μM as final concentration) and CbFDH (16 μM as final concentration) were added. Quickly the resulting mixture was
poured into the 3D-printed mold and allowed to cool down and solidify. Finally, the reactor module was removed from the mold, rehydrated by incubating them in substrate-free reaction buffer for at least 10 min and then placed into the reactor case. After assembling the reactor, the reaction chamber was initially quickly filled with reaction buffer.

3.4.7.3 Activity test of agarose-based flow reactor

Agarose-based hydrogel bricks containing entrapped LE-AmDH-v1 and CbFDH (90 and 16 μM) were prepared as reported in section 3.4.7.2. Reaction conditions: 1 ml final volume in 2 ml Eppendorf tubes; ammonium formate buffer (750 mM, pH 8); NAD⁺ (1mM); 1a (10 mM) as substrate. Entrapped LE-AmDH-v1 and CbFDH were prepared as reported above. The biotransformations were incubated at 170 rpm and 40 °C in an orbital shaker for 24 h. After reaction time was up, 500 μl of the reaction mixture was stopped with 70 μl of 10 M KOH, extracted once with 650 μl of EtOAc containing 10 mM toluene as internal standard (1 min vortexing + 5 min centrifugation, 4 °C, 14800 rpm) and dried over MgSO₄. Furthermore, the second half of the reaction mixture (500 μl) containing also the agarose-based hydrogel brick was extracted with the same procedure described above. Analytical yields were determined by GC-FID (see paragraph Analytics) using toluene as internal standard. Reactions were performed in duplicate.

3.4.8 Recycling of agarose-based reactor in batch

Agarose-based hydrogel bricks containing entrapped LE-AmDH-v1 and CbFDH (90 and 16 μM) were prepared as reported in section 3.4.7.2. Reaction conditions: 1 ml final volume in 2 ml Eppendorf tubes; ammonium formate buffer (750 mM, pH 8); NAD⁺ (1mM); 1a (10 mM) as substrate. Entrapped LE-AmDH-v1 and CbFDH were prepared as reported above. The biotransformations were incubated at 170 rpm and 40 °C in an orbital shaker for 24 h. After reaction time was up, 500 μl of the reaction mixture was stopped with 70 μl of 10 M KOH, extracted once with 650 μl of EtOAc containing 10 mM toluene as internal standard (1 min vortexing + 5 min centrifugation, 4 °C, 14800 rpm) and dried over MgSO₄.
The agarose-based hydrogel brick was then removed from the Eppendorf tube and put in a new one and incubated again for 24 h (this procedure was repeated for 7 days). Between the 3rd and the 4th cycle, agarose-based hydrogel bricks were left over weekend in the fridge and kept hydrated with substrate-free reaction buffer. Finally, in the last cycle, also the second half of the reaction mixture (500 μl) containing the agarose-based hydrogel brick was extracted with the same procedure described above. Analytical yields were determined by GC-FID (see paragraph Analytics) using toluene as internal standard. Reactions were performed in duplicate.

Table 3.3. Recycling test of entrapped LE-AmDH-v1 and CbFDH in agarose-based hydrogel. Conditions: ammonium formate (750 mM pH 8, NAD+ 1 mM), 1a (10 mM), 1 cm x 1 cm gel brick with LE-AmDH-v1 (90 μM) and CbFDH (16 μM) incubated for 24 h at 40 °C and 170 rpm.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Overall age of the reactor [day]</th>
<th>Analytical Yield of 1b (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>New</td>
<td>49 ± 13</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>13 ± 2</td>
</tr>
</tbody>
</table>

### 3.4.9 Reductive amination in flow

Agarose-based flow reactor containing entrapped LE-AmDH-v1 and CbFDH (90 and 16 μM) was prepared as reported in section 3.4.7.2. This led us to obtain a longer reactor (1 cm x 6 cm, diameter x length) with several tunnels inside. After removing the solidified reactor from the mold, this was entered inside an empty HPLC-column (1 cm x 10 cm, diameter x length), and the assembled reactor was manually filled with reaction buffer (ammonium formate, pH 8, 750 mM).

The assembled flow reactor (Figure 3.11) was subsequently mounted on a KD Scientific KD 100 syringe pump unit equipped with a Terumo plastic syringe (10 ml) containing the reaction mixture (10 ml). The flow rate was set at 0.02 ml min⁻¹ and the column was heated up (50 °C) by using a warm water bath. Reactor outcome was collected in 15 ml Falcon tubes. Afterwards, 500 μl of the outflow were mixed with 70
μl of 10 M KOH, extracted once with 650 μl of EtOAc containing 10 mM toluene as internal standard (1 min vortexing + 5 min centrifugation, 4 °C, 14800 rpm) and dried over MgSO₄. Analytical yields were determined by GC-FID (see paragraph Analytics) using toluene as internal standard.

Reaction mixture: 10 ml final volume; 1a (10 mM) in ammonium formate buffer (750 mM, pH 8); NAD⁺ (1 mM).

Reaction resulted in 95.8% analytical yield of 1b in eluted fraction.

Figure 3.10. Flow setup used for the in-flow reductive amination.
3.4.10 Analytics

GC-FID was performed on an Agilent 7890B chromatograph using H$_2$ as carrier gas

Columns: Agilent J&W HP-5 (30 m, 320 μm, 0.25 μm)

Method: T injector 250 °C; constant pressure 6.9 psi; temperature program: 60 °C, hold 6.5 min; 20 °C min$^{-1}$ to 280 °C, hold 1 min.

Retention time
- IS (toluene) – 2.7 min (method B);
- co-solvent (DMSO) – 3.1 min (method B).

Table 4. List of retention times, type of column and acquisition method used for GC analysis

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>Column</th>
<th>Split ratio</th>
<th>Retention time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Benzaldehyde</td>
<td>HP-5-30m</td>
<td>20</td>
<td>4.5</td>
</tr>
<tr>
<td>1b</td>
<td>Benzylamine</td>
<td>HP-5-30m</td>
<td>20</td>
<td>5.1</td>
</tr>
</tbody>
</table>
3.5 References

(3) Gomollón-Bel, F. Chemistry International 2019, 41, 12.