Valency Engineering of Monomeric Enzymes for Self-Assembling Biocatalytic Hydrogels

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Supplementary Information

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Materials and Methods

Plasmid construction:

Unless otherwise stated, the genetic construction was carried out using the isothermal recombination as described by Gibson *et al.* utilizing oligonucleotide primers with 20-30 bp homologous overlaps.[1] After the assembly, the reaction mixtures were treated with DpnI to remove any remaining vector from prior PCR reactions and then transformed into *E. coli* DH5α cells. All plasmids were purified using ZR Plasmid Miniprep– Classic (Zymo Research, Germany) according to the manufacturer's instructions. Sequences were verified by commercial sequencing (LGC genomics, Germany).

pET_GDH-$(GGGGS)_2$-SC-G-His:

The backbone encoding for a N-terminal GDH-GGGGS and C-terminal HisTag separated by a glycine spacer was amplified utilizing primers SG25 and SG47 with pET_GDH-$(GGGGS)_2$-ST-G-His as the template.[2] This backbone was then recombined with a SC encoding insert, which had been generated by PCR using the primers SG48 and SG29 with pTF16_Lpp-OmpA-SC[3] as the template.

pET_ST-GGGGS-Gre2p-GGGGS-ST-G-His:

The N-terminal ST sequence and a flexible GGGGS linker sequence were inserted into plasmid pET_ Gre2p-GGGGS-ST-G-His [4] by PCR using the primers SG50 and SG49.

pET_ST-GGGGS-Gre2p(F86-GGGGS-ST-GGGGS-D87)-GGGGS-ST-G-His:

The plasmid pET_ST-GGGGS-Gre2p-GGGGS-ST-G-His was amplified by PCR using the primers IRG1 and IRG2, resulting in a plasmid backbone opened between the amino acid positions F87 and D87 of Gre2p. Two complementary single stranded oligonucleotides containing the sequence for GGGGS-ST-GGGGS (synthesized by Sigma Aldrich) were hybridized by incubating an equimolar ratio at 95°C for 5 minutes and subsequently slow cooling to room temperature. The resulting double stranded sequence was recombined with the opened backbone by Gibson assembly, as described above. Plasmids for expression of Gre2p with alternative positions of internal ST were cloned similarly by using the Primers IRG3 und IRG4 for pET_ST-GGGGS-Gre2p(K139-GGGGS-ST-GGGGS-S140)-GGGGS-ST-G-His, IRG5 und IRG6 for pET_ST-GGGGS-Gre2p(E228-GGGGS-ST-GGGGS-D229)-GGGGS-ST-G-His and IRG7 and IRG8 for pET_ST-GGGGS-Gre2p(S296-GGGGS-ST-GGGGS-T302)-GGGGS-ST-G-His.

PCR primers used in this study.

SG25	GGTCATCATCACCATCACCATTGAG
SG47	GCTACCACCACCACCGCTAC
SG48	GGACGCGGTGGTGGTGGTGGTAGCGGTGGTGGTGGTAGCGTTGATAC
SG29	TCAAACTCAATGGTGATGGTGATGATGACCAATATGTGCATCACCTTTGGTTGC
SG50	TGGTTGACGCTTACAAACCGACCAAAGGTGGTGGTGGTAGCTCAGTTTTTGTTTCAG GTGCTAACGGGTTC
SG49	CGGTTTGTAAGCGTCAACCATAACGATGTGAGCCATATGTATATCTCCTTCTTAGTAC AAACTTGTGATATTC
IRG1	CCAAAGGCGGTGGCGGTAGCGATATCACTGACAGTGAACGCGATTTATTAATTC
IRG2	TGAGCGCTGCCACCGCCACCAAAGCAGAATGGAGAGGCCGTATGTAG
IRG3	CCAAAGGCGGTGGCGGTAGCTCTTTAACATTTAACGAAGAATCCTGGAACCC
IRG4	TGAGCGCTGCCACCGCCACCTTTATCGTTTTCTTTTGCCATGTCGAACACAG
IRG5	CCAAAGGCGGTGGCGGTAGCGACAAGATACCGGAACTATTTGGTGGATAC
IRG6	TGAGCGCTGCCACCGCCACCCTCTGGTGATAAATGCATCAAGCTGTTGAC
IRG7	CCAAAGGCGGTGGCGGTAGCACCCTTGGTGCTACTCTTGATAATAAAAAGAG
IRG8	TGAGCGCTGCCACCGCCACCAGAACCTGGTTTCCCCACTGGAATATTG

Protein expression and purification

E. coli BL21 (DE3) cells were transformed with the corresponding expression vector using heat shock transformation. Transformed E. coli cells harboring the various plasmids were selected overnight on LB/agar plates containing 100 µg/ml ampicillin at 37°C. Liquid cultures of 160 ml LB medium containing ampicillin were generated from clones of the LB/agar plates cultured overnight for 14-18h at 37 °C, 180 rpm in a 500 ml shaking flask. 2 L ampicillin-containing LB-medium was inoculated 1:20 with overnight culture. The cultures were incubated at 37°C, 180 rpm until an OD600 of 0.6 was reached. The temperature was then lowered to 25°C, IPTG was added to a final concentration of 0.1 mM and the cultures were incubated for an additional 16 hours. The cultures were pooled and cells were harvested by centrifugation (10000xg, 10 min, 4°C) and resuspended in 60 mL buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.0). After disruption by ultrasonication, the cell lysate was centrifuged (45000xg, 1 h, 4°C) and the supernatant was filtered through a 0.45 µm Durapore PVDF membrane (Steriflip, Millipore) and loaded on two HisTrap FF (5 mL) Ni-NTA columns (GE Healthcare, Germany) connected in series and mounted on an Äkta Pure liquid chromatography system (GE Healthcare, Germany). The columns were washed with 100 mL buffer A and the 6xHis-tagged proteins were eluted using a gradient from 100% buffer A to 100% buffer B (50 mM NaH_2PO_4,

300 mM NaCl, 500 mM Imidazole, pH 8.0) over a volume of 200 ml. Subsequently, the buffer was exchanged to KPi-Mg (100 mM KP_i pH 7.5, 1 mM $MgCl_2$) using Vivaspin 10 000 MWCO (GE Healthcare).

To characterize the recombinant, purified proteins, samples were typically analyzed by standard discontinuous SDS-polyacrylamide Laemmli-midi-gels. The bands were visualized by Coomassie staining and compared to the Color Prestained Protein Standard, Broad Range (New England Biolabs). The concentrations were determined by UV-Vis spectroscopy, using the theoretical molar extinction coefficients at 280 nm, as calculated by the Geneious version 9.1.3 software.[5].

Dynamic light scattering measurements

The changes in hydrodynamic diameter upon polymerization was determined over time by dynamic light scattering (DLS) analysis, using the Nano-Series ZetaSizer (Zetasizer Nano ZSP, Malvern Instruments), equipped with a He-Ne-Laser (633 nm). Prior to DLS measurement, all protein solutions were preincubated in a thermoshaker for 5 minutes at the temperature of the following measurement and 500 rpm. Subsequently, if not stated otherwise, two protein solutions (500 µM) were mixed in a UV-cuvette at variable molar ratios (1:9; 1:3; 1:2; 1:1; 2:1; 3:1; 9:1) at a total volume of 100 µL. Samples were incubated at 500 rpm in a thermoshaker in between the measurements. The increase of the average hydrodynamic radius of the protein particles (Z-Average) within the first 30 min of SC-ST polymerization were calculated from auto-correlated light intensity data using the ZetaSizer Software. Typically, DLS measurements were carried out at 25 °C over 2 h with individual measurements every 90 s.

Hydrogel preparation

Protein solutions of either GDH-SC-His/Gre2p-ST_2, Gre2p-ST_3 to yield hydrogels, or GDH-SC-His/Gre2p-His used as negative control, were diluted in KP_i-Mg (100 mM KP_i pH 7.5, 1 mM $MgCl_2$) to a final concentration of 500 µM in 20 µl. Polymerization was carried out for 1 h at 30°C, 1000 rpm in a thermoshaker. Subsequently, the buffer was evaporated from a 0.2 ml reaction tube with an open lid under constant centrifugation at 2200xg for 15-17 h at 30°C. For MPT analysis, the samples were supplemented with 0.2 mg/ml ‘dragon’ green fluorescent polystyrene microspheres (200nm diameter; Bangs Laboratories, USA).

Optical microrheology based on multiple particle tracking (MPT)

Prior to MPT-analysis, the dried hydrogel samples were swollen by adding 10 µl KP_i-Mg for 10 minutes under continuous shaking at 25°C, 500 rpm. MPT experiments were performed using an inverted fluorescence microscope (Axio Observer D1, Zeiss), equipped with a Fluar 100x, N.A. 1.3, oil-immersion lens combined with a 1x optovar magnification changer. The Brownian motion of more than 100 green fluorescent polystyrene microspheres of 200 nm diameter used as tracer particles was tracked. Images of these fluorescent beads were recorded using a sCMOS camera Zyla X (Andor Technology, UK). Displacements of particle centers were monitored in an 127 x 127 µm field of view, at a rate of 50 frames/s. Movies of the fluctuating microspheres were analyzed using a custom MPT routine incorporated into the software Image Processing System (Visiometrics iPS) and a self-written Matlab program[1] based on the widely used Crocker and Grier tracking algorithm[6].

To characterize sample heterogeneities, the distribution and slopes of the MSDs were analyzed. Further, the van Hove correlation functions[7], i.e., the probability distribution of particle displacement for an ensemble of N tracked particles, were calculated as:

$$P(x,\tau) = \frac{1}{N}\left\langle \sum_{i=1}^{N} \delta\left[x + x_i(0) - x_i(\tau)\right]\right\rangle = \frac{N(x,\tau)}{N}$$

Where $N(x,\tau)$ is the number of particles found at positions between x and x + dx along the x-coordinate. $P(x,\tau)$ is Gaussian if all tracer particles are exposed to a similar environment. Deviations from this functional form reflect the presence of spatial heterogeneities and can be characterized by the non-Gaussian parameter α [8].

$$\alpha = \frac{\left\langle x^4(\tau)\right\rangle}{3\left\langle x^2(\tau)\right\rangle^2} - 1$$

This quantity is zero for a Gaussian distribution, while deviations can result in large α values.

Microreactor preparation

The microfluidic reactor was prepared as previously described.[2] In brief, the reactor designs were based on the dimension of standard microscope slides (76 x 26 mm² DIN ISO 8037-1:2003-05). The upper part containing the reaction channel was manufactured by replica casting of polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, USA) in brass replication molds. The straight channel was 3 mm wide, 1 mm high and 54 mm long with a total volume of 150 µL. Cannulas (Sterican, B. Braun Melsungen AG, Germany) were inserted through horizontal holes in the molds before pouring the PDMS prepolymer to serve as placeholders for the cannulas. The PDMS was cured at 60 °C for at least 3 h.

Synthesis and racemic reduction of methylketones

Acetophenone and 4′-chloroacetophenone were purchased from Sigma Aldrich. Synthesis and characterization of NDK **1** was performed as previously described.[9] For synthesis of racemic reduction products, one equivalent ketone (>50 mg) was dissolved in MeOH (5 mL) and two equivalents $NaBH_4$ were slowly added under stirring at room temperature. Products were partitioned between water and ethyl acetate and the organic phase was evaporated.

Chiral HPLC analysis

An Agilent 1260 series HPLC equipped with a Diode Array Detector and a Lux 3µ Cellulose-1 (150x2.00 mm) chiral column (Phenomenex) was used for chiral analysis. The analysis of biocatalytic NDK reaction products by chiral HPLC was performed as previously described.[9] For the reaction analysis of **1**, the dried ethyl acetate extractions from the crude reaction mixtures (described above) were resuspended in 100 µl of the mobile phase (90% n-heptan, 10% 2-propanol) and 10 µl of that solution were injected into the HPLC instrument. The hydroxyketones **2** were analyszd by method A, diols **3** by method B, as detailed below. Reduction of acetophenone **4**, leading to (*S*)-**5** or (*R*)-**5b**, was analyzed by method C. Reduction of 4'-chloroacetophenone **6**, leading to (*S*)-**7** and (*R*)-**7b** was analyzed by method D. In method C and D, 30 µl of the crude reaction mixture were injected into the HPLC instrument. An overview of the methods is shown below:

Overview of the used chiral HPLC methods used in this study.

Method	A	B	C	D
mobile phase	90% Heptan 10% 2-propanol	98% Heptan 2% 2-propanol	85% H_2O 15 % ACN	80% H_2O 20 % ACN
flowrate [ml/min]	0.5	1.0	0.4	0.4
column oven [°C]	10	45	40	40
detection wavelength [nm]	210	210	210	210
retention time [min]	$R_{t\ (3\text{-}d)}$ = 3 – 3.5 $R_{t\ (2b)}$ = 3.9 $R_{t\ (2c)}$ = 4.5 $R_{t\ (2d)}$ = 5.0 $R_{t\ (2)}$ = 5.4 $R_{t\ (1)}$ = 6.0	$R_{t\ (1)}$ = 3.8 $R_{t\ (2\text{-}d)}$ = 6.7 – 9.1 $R_{t\ (3)}$ = 12.4 $R_{t\ (3c)}$ = 14.1 $R_{t\ (3d)}$ = 14.8 $R_{t\ (3b)}$ = 16.3	R_t (*(R)-1-Phenylethanol* **5b**) = 6.4 R_t (*(S)-1-Phenylethanol* **5**) = 7.0 $R_{t\ (acetophenone\ 4)}$ = 13.2	$R_{t\ (chloroacetophenone\ 6)}$ = 6.3 R_t ((R)*-4-Chloro-α-methylbenzyl alcohol* **7b**) = 11.3 R_t (*(S)-4-Chloro-α-methylbenzyl alcohol* **7**) = 10.8

Determination of enzymatic activity

The specific activities of the enzymes were determined as previously described.[4] In brief, enzyme assays were performed with a reaction mixture containing 5 mM NDK 1, 100 mM glucose, 1 mM $NADP^+$, in KP_i-Mg containing 0.5 µM of the respective tagged Gre2p ketoreductase and an excess of 10 µM GDH-His for NADPH-regeneration. The GDH-containing reaction mixture was preincubated for at least 30 min at 30°C prior to the addition of the ketoreductase. For the determination of the average specific activity, samples for HPLC analysis were taken after 20 min, while for the determination of enzyme kinetics, reactions were carried out for at least 5 h. Samples were taken manually at various time points and were subsequently analyzed by chiral HPLC. In order to measure the GDH activity, 0.5 µM of the respective tagged GDH was incubated together with an excess of 10 µM ketoreductase, using the same conditions as described above for the ketoreductase kinetics. For HPLC analysis, 50 µL of the crude reaction mixtures were extracted with 150 µL ethyl acetate, centrifuged for phase separation, and 75 µl of the organic phase were transferred into HPLC vials and evaporated (Concentrator plus, Eppendorf). Chiral HPLC analyzes were carried out as described above. Values for conversion, enantiomeric and diastereomeric excess were calculated based on the peak intensity of HPLC signals of the substrate and products detected at 210 nm, as previously described.[9]

Microfluidic setup and analysis of the hydrogels under continuous flow

The microreactors were placed inside an incubator (set to 30°C), filled with 150 µl 1000 µM protein solution (GDH-SC/Gre2p-ST_2 or GDH-SC/Gre2p-ST_3, molar ratio 2:1) and incubated for 30 min. The PDMS chips were then sealed with a with a polyolefin foil (HJ-BIOANALYTIK GmbH, Germany). Low pressure syringe pumps (neMESYS 290N) equipped with 5 or 10 mL syringes were connected to a manual switching valve that was connected to the reactor for perfusion of reaction media at a flowrate of 5 µL/min. The syringes were filled with 5-10 mL substrate solution containing 5 mM of substrate (NDK **1**, acetophenone **4** or 4′- chloroacetophenone **6**, respectively), 100 mM glucose in KP_i-Mg, supplemented with 0.01 % (v/v) sodium azide to avoid fouling and 1 mM $NADP^+$. The reactor outflow was connected to the Compact Positioning System rotAXYS or rotAXYS360 (CETONI, Germany) to allow for automatic fractioning into wells of 96-well plates, previously loaded with 50 µl 7 M CH_5N_3 HCl to stop all enzymatic reactions. The positioning system was connected to a CETONI neMESYS Base module, which was controlled by the QmixElements-Software. The chip was connected to syringes and the fraction collector by tubings (Inlets: silicone Tygon tubing R3603, ID = 1.6 mm, Saint-Gobain, France; outlets: conventional PTFE tubing, ID = 0.5 mm) using standard cannulas and luer lock fittings.

Preparative synthesis and purification of the biocatalytic reduction products of NDK 1

General

Analytical thin layer chromatography (TLC) was performed on ALUGRAM® SIL G / UV254 plates (Macherey-Nagel), while column chromatography was performed using Roth Silica gel 60 (230–400 mesh). TLC plates were stained with ninhydrin (1.5 g dissolved in 100 mL ethanol) for analysis.

Procedure

The biocatalytic reduction of NDK 1 (300 mg, 1.5 mmol) was carried out with stack of six microreactors, as described above. The aqueous product mixture collected from the outflow was extracted with EtOAc (3x equal reaction volume), the organic phase was washed with brine, dried over magnesium sulfate and evaporated. The residue was purified by column chromatography (SiO_2, gradient n-Hex/EtOAc = 2:1 to EtOAc). The product containing fractions were combined to yield (S)-Hydroxyketone 2 (141 mg, 0.69 mmol, 46 %, Rf = 0.22) and (S,S)-Diol 3 (83 mg, 0.41 mmol, 27 %, Rf = 0.10). NMR data were according to previously reported spectra.9

Supporting Figures

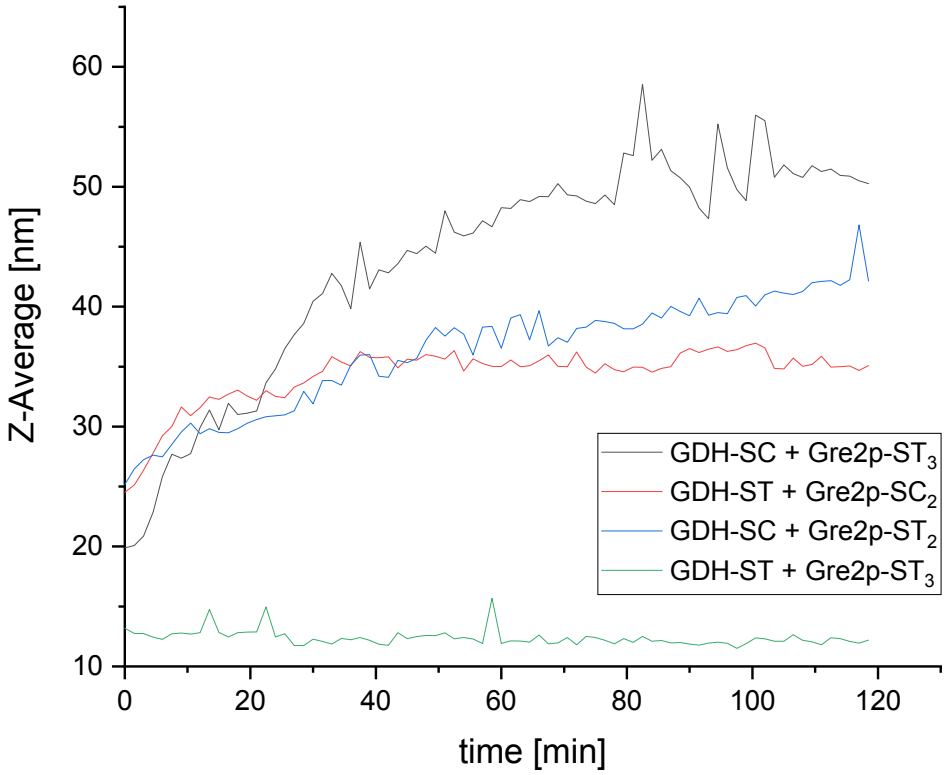
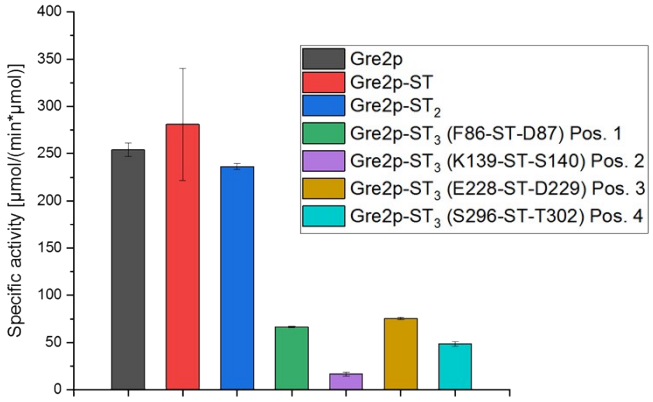


Figure S1: Increase of the hydrodynamic particle diameter determined for different compositions of potentially hydrogel-forming enzyme variants, determined at 25 °C over 2 h by DLS measurements. Note that the increase in hydrodynamic particle diameter is clearly visible for all combinations except of the control (green line), thereby providing clear evidence for the polymerization reaction. For all measurements 1 mM solutions of the corresponding enzymes were mixed in a binding site ratio of 1:1.

a



b

Protein	Yield mg/$L_{culture}$
Gre2p	45
Gre2p-ST	37
Gre2p-ST_2	35
Gre2p-ST_3 Pos. 1	48
Gre2p-ST_3 Pos. 2	< 0,1
Gre2p-ST_3 Pos. 3	2
Gre2p-ST_3 Pos. 4	3

Figure S2: Specific activities (a) and expression yields (b) of the various ST-tagged Gre2p variants.

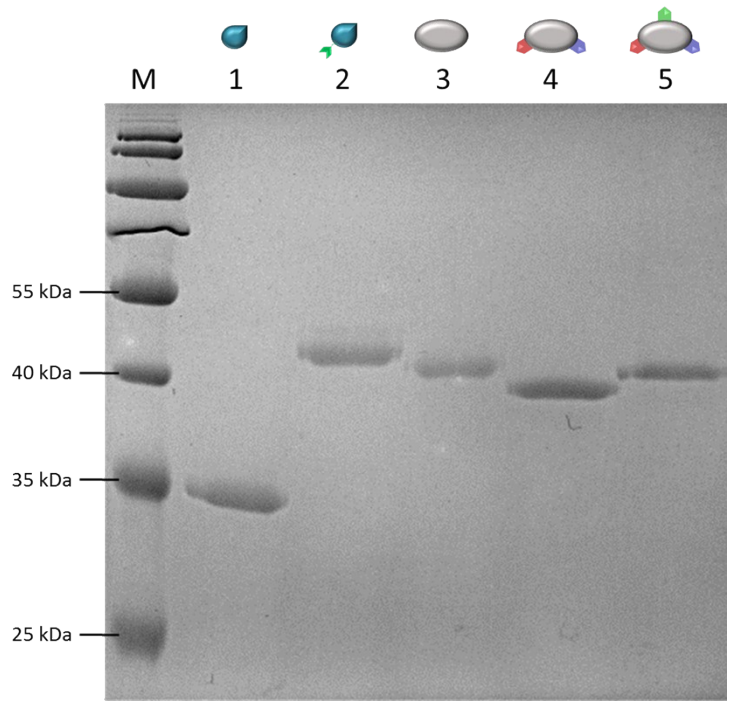


Figure S3: SDS gel electrophoretic analysis of purified proteins, used in this study. This is a coomassie stained 15 % SDS-PAGE. M: Color Prestained Protein Standard, Broad Range (New England Biolabs); lane 1: BsGDH-His (29 kDa); lane 2: BsGDH-SC-His (41 kDa); lane 3: Gre2p-TEV-His (43 kDa); lane 4: Gre2p-ST_2-His (42 kDa); lane 5: Gre2p-ST_3-His (44 kDa). Note that a previously described and slightly larger Gre2p-TEV-His (lane 3) variant that contains a C-terminal TEV protease cleavage site [9] was used for comparison of the enzymatic activity of double (lane 4) and triple (lane 5) tagged Gre2p variants.

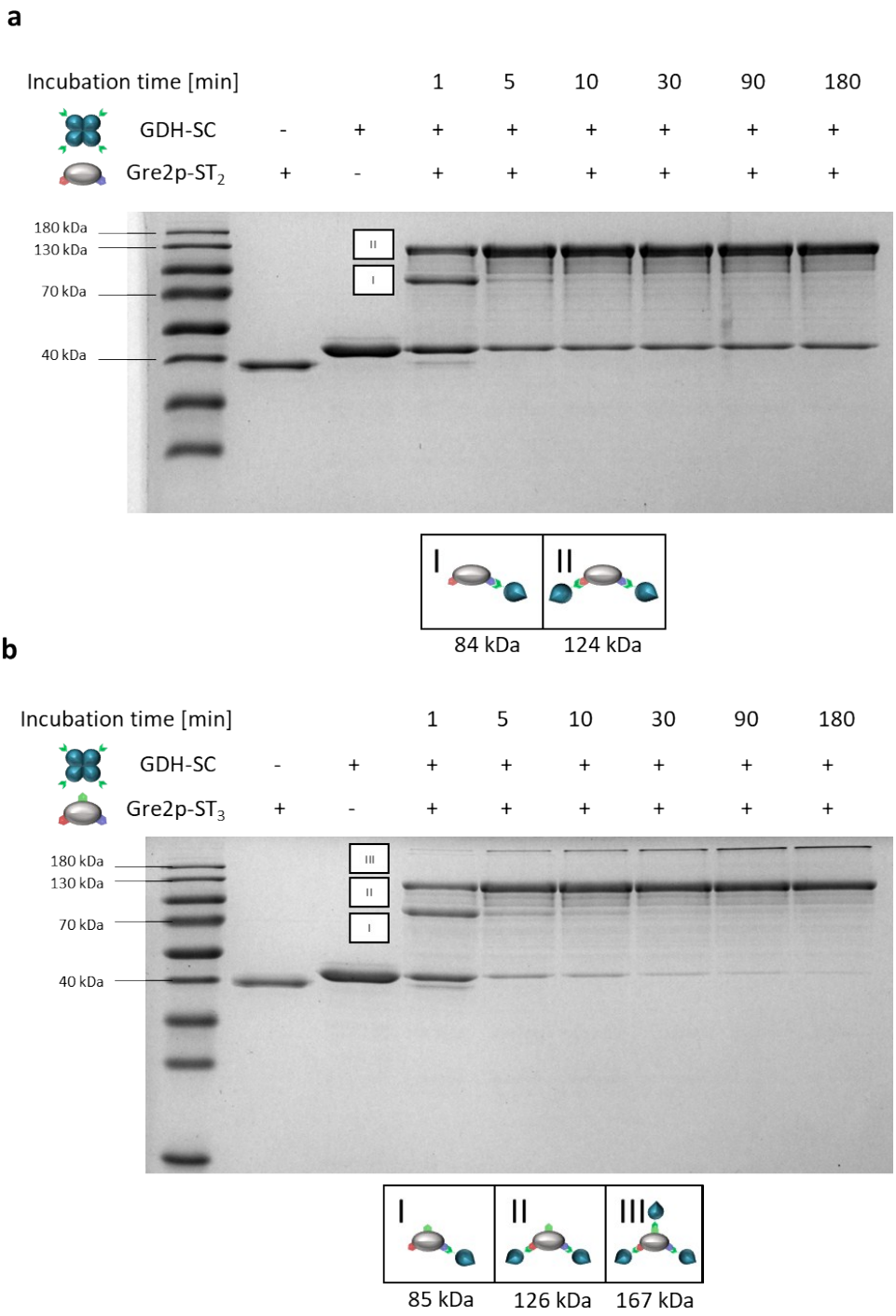


Figure S4: Kinetic analysis of ST/SC-mediated conjugation of GDH-SC with (a) Gre2p-ST_2 or (b) Gre2p-ST_3, respectively (Coomassie stained 15 % SDS-PAGE; 36 pmol of (a) GDH-SC : Gre2p-ST_2 mixed in a molar subunit ratio of 2:1) or (b) GDH-SC : Gre2p-ST_3 mixed in a molar subunit ratio of 2:1; incubation at 30°C and 1000 rpm. The reaction was stopped by addition of SDS-loading dye and heating to 95°C for 10 minutes to denature non-covalent protein interactions. Schemes underneath illustrate the expected conjugates. Note that in the case of double tagged Gre2p-ST_2, two distinct crosslinked protein species of 84 kDa and 124 kDa were formed, whereas triple tagged Gre2p-ST_3 leads to three crosslinked protein species of 85 kDa, 126 kDa and > 180 kDa. Hence, the observed product formation indicates that crosslinking occurs at all two or three tag positions, respectively. However, unreacted GDH-SC is still present in the case of Gre2p-ST_2, whereas Gre2p-ST_3 shows that nearly all subunits are crosslinked with two or three GDH monomers.

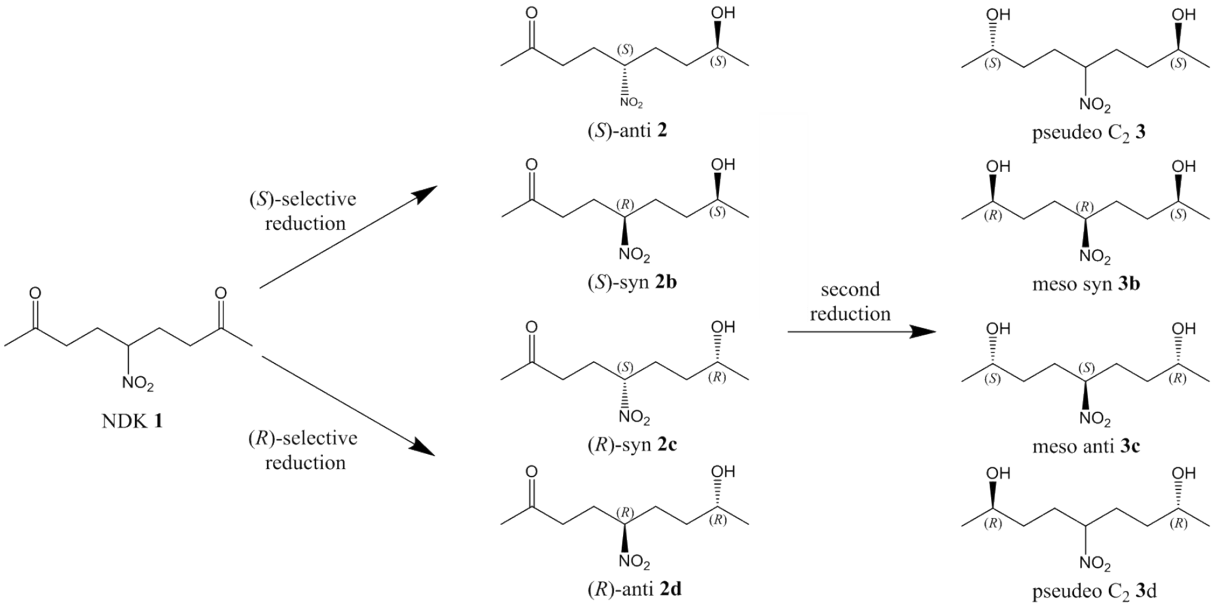
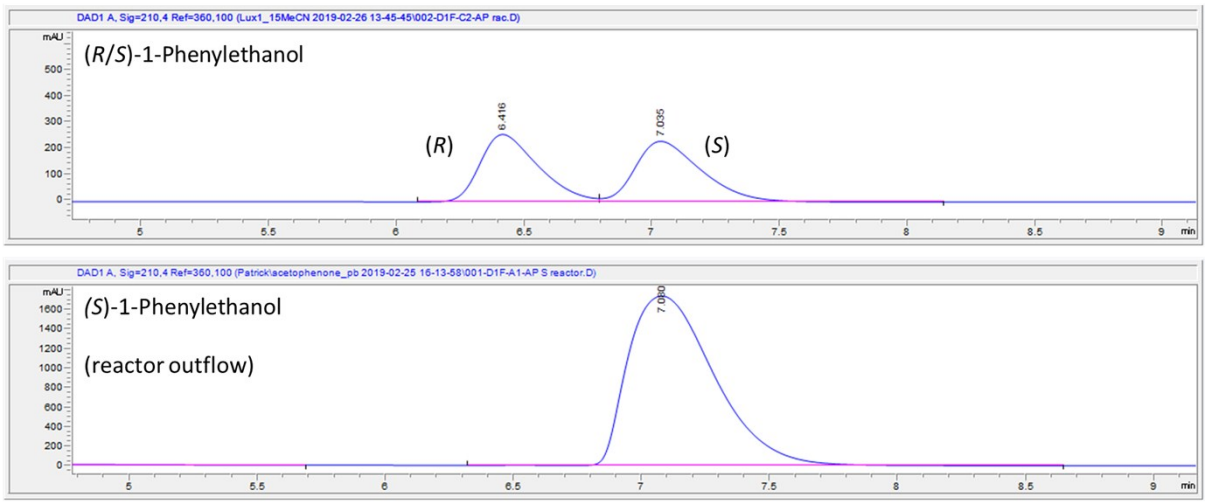


Figure S5: Reaction scheme of the reduction of NDK **1**. The prochiral C_S-symmetrical 5-nitrononane-2,8-dione (NDK) **1**can be reduced, depending on the enzyme selectivity, either on one or on both of the two carbonyl groups to create the hydroxyketones **2** or diols **3**, respectively. All stereoisomers can be identified using by chiral HPLC.

a



b

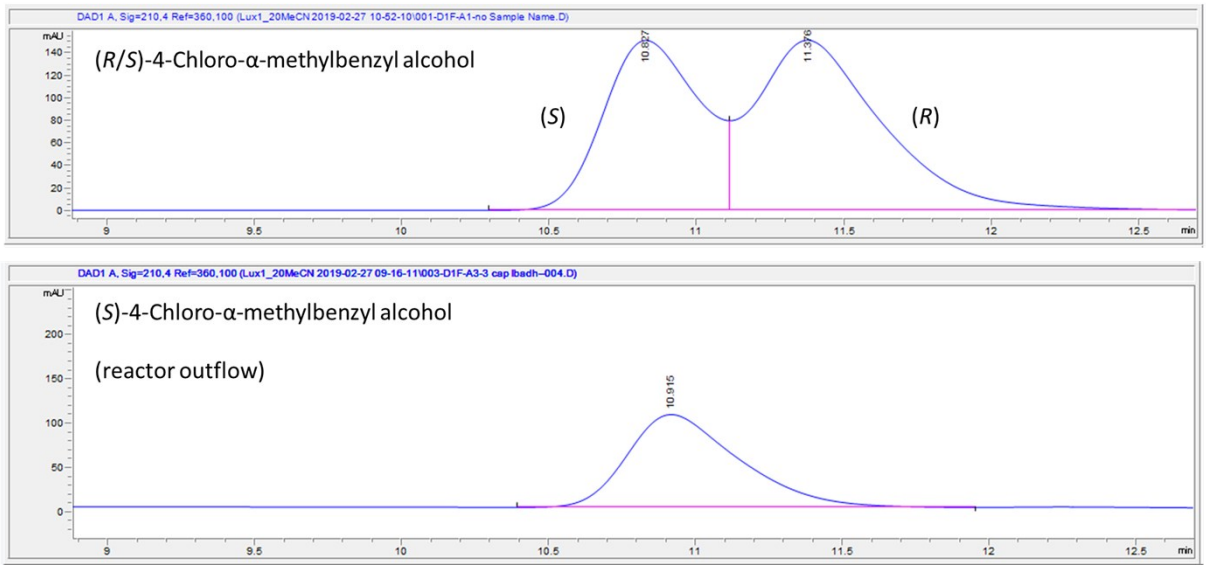


Figure S6: Representative HPLC traces of the corresponding alcohols obtained from chemical reduction of a) acetophenone and b) 4-chloroacetophenone. The traces below show the result of the analysis of the outflows of the (S)-hydrogel reactors. HPLC traces obtained for the conversion of NDK were similar as reported previously.[9]

a

b

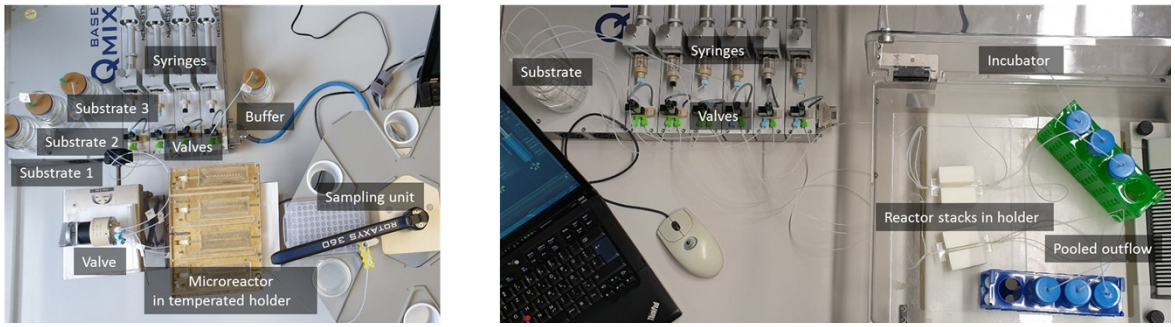


Figure S7: Microfluidic setup for continuous flow biocatalysis. Overview of the microfluidic system used for (a) sequential perfusion of different methylketones (data shown in Figure 3) or (b) for the 'numbering up' experiment (data shown in Figure 4b).

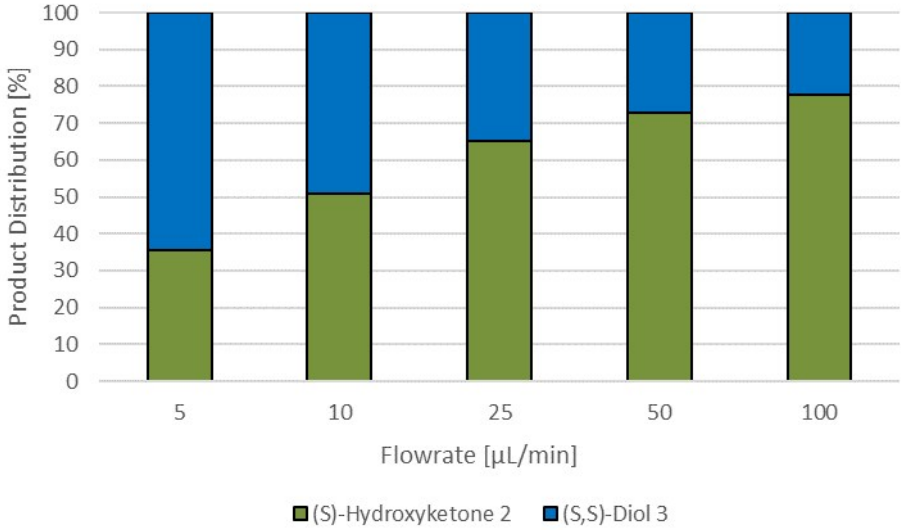


Figure S8: Flowrate dependent distribution of the products hydroxyketone **2** and diol **3**. Note that with increasing flowrate the product ratio is shifted towards the intermediate hydroxyketone **2**.

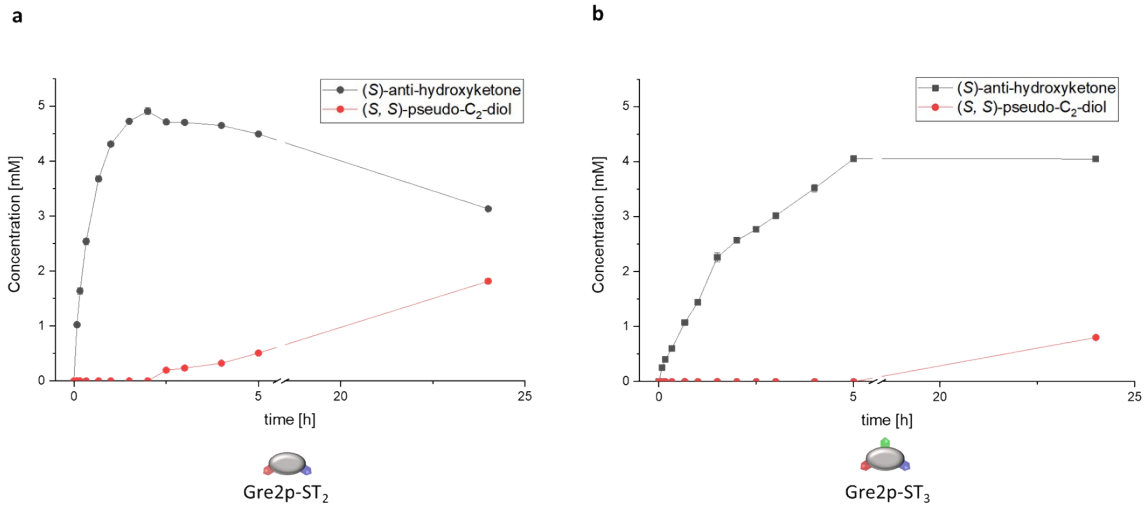


Figure S9: Time dependent stereoselective conversion of NDK **1** by the (*S*)-selective Gre2p-ST_2 (a) or Gre2p-ST_3 (b), respectively, to (*S*)-anti hydroxyketone **2** (black) which is slowly reduced to (*S*,*S*)-configured pseudo C_2 **3** (red). Note that Gre2p-ST_2 is about 3.6-fold more active than Gre2p-ST_3.

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