Supporting Information

A 3D printable synthetic hydrogel as an immobilization matrix for continuous synthesis with fungal peroxygenases

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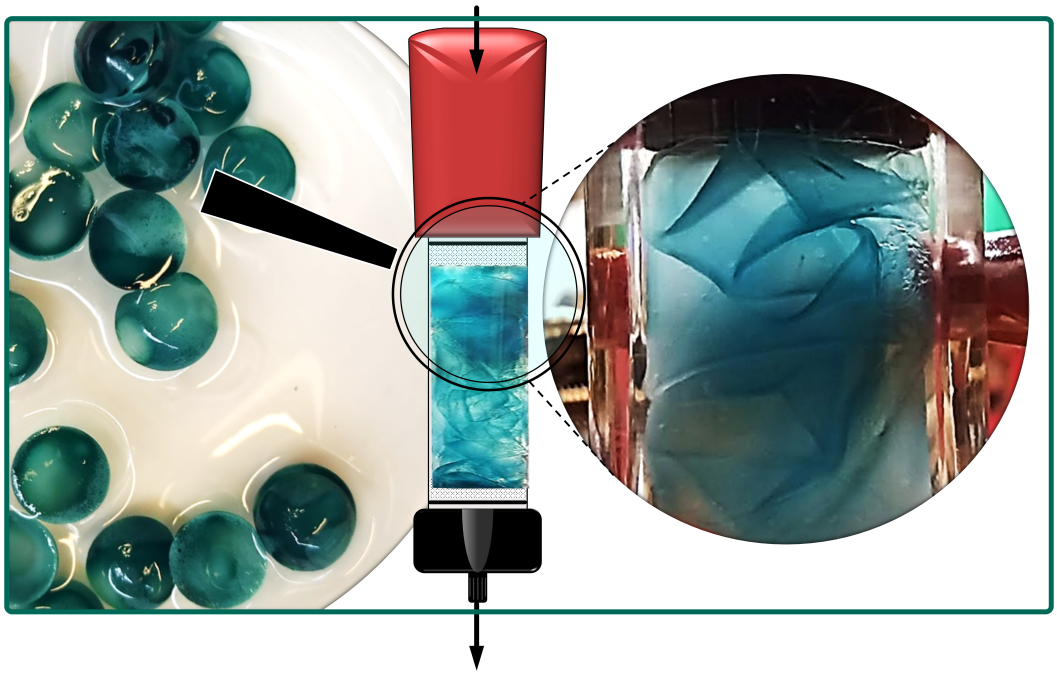
Development of a novel immobilization strategy using synthetic 3D printable hydrogels for flow biocatalysis



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1 Materials and methods

1.1 General information

All solvents, reactants, and starting materials were received from commercial suppliers in the highest available purity (Sigma-Aldrich, VWR) and used as received. Ultrapure water (UPW, 18.2 MΩ·cm) was produced with a Milli-Q® Synthesis system by Millipore Corporation (now Merck Millipore, Darmstadt, Germany) and used throughout this study. All experiments were carried out under atmospheric conditions if not stated otherwise.

Potassium phosphate buffer solution (KPi, 50 mmol·L^{-1}, pH 7.0) was prepared by adding 0.0268 mol·L^{-1} (4.67 g·L^{-1}) of dipotassium hydrogenphosphate (K_2HPO_4) and 0.0232 mol·L^{-1} (3.16 g·L^{-1}) of potassium dihydrogenphosphate (KH_2PO_4) to UPW. The pH was always checked after final preparation with a FiveEasy pH meter F20 from Mettler-Toledo GmbH (Gießen, Germany).

Unspecific peroxygenase (UPO) mutant PaDa-I was expressed in *Pichia Pastoris*, and a DASGIP® Parallel Bioreactor System with Bioblock from Eppendorf (Hamburg, Germany) was used to produce the enzyme. The detailed protocol can be found elsewhere.[1] The PaDa-I solution is a crude extract supernatant preparation achieved after fermentation.

Volumetric activity of free PaDa-I was determined via standard 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay (single oxidation of ABTS to $ABTS^{\cdot+}$ radical, see Scheme 1). ABTS solution was prepared in UPW by adding 0.0882 mol·L^{-1} (15.7 g·L^{-1}) of disodium hydrogenphosphate dihydrate ($Na_2HPO_4 \times 2\ H_2O$) and 0.0559 mol·L^{-1} (11.8 g·L^{-1}) of citric acid resulting in a final pH of 4.4. Eventually, 0.3 mmol·L^{-1} of ABTS ($C_{18}H_{16}N_4O_6S_4$-$(NH_4)_2$, M = 548.7 g·mol^{-1}, 0.166 g·L^{-1}) were added. The pH was always checked after final preparation with a FiveEasy pH meter F20 from Mettler-Toledo GmbH (Gießen, Germany).

For diffusion experiments, seven blank hydrogel pellets were washed in KPi containing basic fuchsin dye in a beaker with stirring at 300 rpm for 0.5, 1, 5, 10, 15, 30, and 60 minutes. The pellets were then cut in half and the deepness of the colour throughout the whole pellet was optically examined.

1.2 Immobilization of PaDa-I in AETMA-PEGDA 700 hydrogels

1.2.1 Synthesis of blank hydrogels

1.216 g [2-(acryloxy)ethyl]trimethyl ammonia chloride (AETMA) and 0.336 g (22 wt%) polyethyleneglycol diacrylate (PEGDA 700) were added to a vial. In another vial, 0.0033 g lithium phenyl-2,4,6,-trimethyl benzoyl phosphinate (LAP) was dissolved in 120 µL purified water and thoroughly mixed. Then, the LAP solution was added to the mixture of monomer (AETMA) and crosslinker (PEGDA 700) and thoroughly mixed at room temperature. 10 µL of this mixture was added to a 1.5 mL microcentrifugation test tube and then irradiated

under a UV-lamp (Analytik Jena US, UVP UVLMS-38 Upland, US) at 365 nm for 3.5 min. Thereafter, the hydrogels were carefully removed from the tube and exposed to white light for 1 h. Afterwards, mass, height and diameter were determined. If necessary, hydrogels were dried on the air for 24 h or 72 h, and the above listed parameters were measured again. The hydrogels were washed in 5 mL KPi buffer (50 mmol·L^{-1}, pH 7.0) for one hour at 80 rpm on a roller mixer from IKA® (Staufen, Germany). The washing was repeated twice and after the third washing step, mass, height, and diameter were determined.

1.2.2 Hydrogel-based immobilization of PaDa-I

The heterogenization of the PaDa-I enzyme into the synthetic hydrogels was performed as described above ("synthesis of blank hydrogels") with a slight change in protocol: instead of using 120 µL purified water, this time, a mixture of 78 µL liquid enzyme (equals to ca. 71 U) and 42 µL purified water was used. After the third washing step, mass, height, and diameter of the pellets were measured again.

Further batches were synthesized to test the effect of certain parameters on the activity of the hydrogel pellets. These parameters were the amount of crosslinker (in wt%), the amount of enzyme immobilized, and the initial volume of the pellets. Two batches, one with 44 wt% (0.869 g AETMA / 0.683 g PEGDA 700), and the other with 11 wt% (1.381 g AETMA / 0.171 g PEGDA 700) of crosslinker were produced. This means double or half amount of PEGDA 700 compared to the standard procedure (22 wt%), thus a denser or wider polymer network, respectively.

A different batch of hydrogels with 120 µL enzyme (equals to 109 U) and without any additional water was prepared to test the activity in case of increased amount of entrapped PaDa-I. Another batch was synthesized with 39 µL PaDa-I (equals to 35 U) and 81 µL purified water, and afterwards an activity assay was performed with two pellets at the same time. Enzyme activity was also determined from an additional batch of 5 µL hydrogels prepared analogously to the standard procedure.

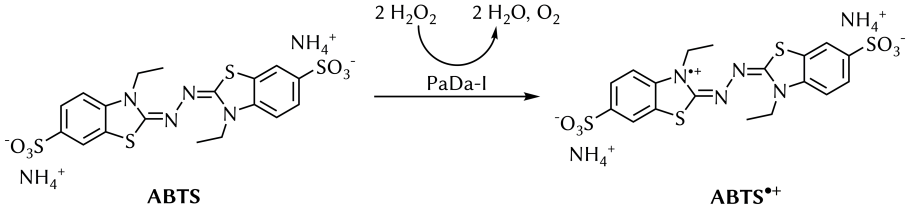
1.3 Assay of free and immobilized PaDa-I

1.3.1 Determination of the activity

One unit (U) of enzyme activity is defined as the amount of enzyme converting 1 µmol substrate per minute under standard assay conditions (Scheme S1). For investigating the free form of the enzyme, 990 µL ABTS solution, 10 µL enzyme dilution and 1.75 µL 3.5% (v/v) H_2O_2 were mixed and continuously measured in a cuvette at 405 nm at room temperature in a UV-1600PC UV/vis spectrophotometer from VWR International (Darmstadt, Germany). Samples were measured in triplicate and the activity was calculated according:

$$A_{\text{vol.}}\left(\frac{\text{U}}{\text{mL}}\right) = \frac{\Delta\text{Abs}_{405} \cdot \text{DF} \cdot V}{\varepsilon_{405\text{ nm}}^{\text{ABTS}^{\bullet+}} \cdot v \cdot d}$$

$\Delta\text{Abs}_{405}\left(\frac{1}{\text{min}}\right)$ = Measured absorbance at 405 nm over time;
$\text{DF}(-)$ = Dilution factor;
$d(\text{cm})$ = Light path;
$V(\text{mL})$ = Sample volume;
$v(\text{mL})$ = Enzyme volume in the sample;
$\varepsilon_{405\text{ nm}}^{\text{ABTS}^{\bullet+}}$ = Molar extinction coefficient, $36.8 \times 10^3\ \text{mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$.



Scheme S1. Oxidation of ABTS to its radical form $\text{ABTS}^{\bullet+}$.

To investigate the hydrogel-entrapped enzyme, 10 mL of 0.3 mmol·L^{-1} of ABTS and the immobilized enzyme were stirred at 300 rpm at 25 °C. From the time of the addition of 17.5 µL of 3.5% (v/v) H_2O_2, five samples (ca. 0.5 mL each) were taken every 30 seconds and measured at 405 nm at room temperature. The activity assay was performed from the freshly immobilized enzyme, furthermore, experiments were carried out to describe the storability and reusability. All measurements were executed also with a reference hydrogel without enzyme. The experiments were performed in triplicate and the specific activity and activity yield were calculated according to:

$$A_{\text{spec.}}\left(\frac{\text{U}}{\text{g}_{\text{wet carrier}}}\right) = \frac{\Delta\text{Abs}_{405} \cdot \text{DF} \cdot V}{\varepsilon_{405\text{ nm}}^{\text{ABTS}^{\bullet+}} \cdot m_{\text{wet carrier}} \cdot d}$$

$$\text{Activity yield}\ (\%) = \frac{A_{\text{spec.}}\left(\frac{\text{U}}{\text{g}_{\text{wet carrier}}}\right) \cdot \text{m}\left(\text{g}_{\text{wet carrier}}\right)}{A_{\text{vol.}}\left(\frac{\text{U}}{\text{mL}}\right) \cdot V_{\text{stock}}(\text{mL})}$$

where V_{stock} is the enzyme volume used for the preparation of only one hydrogel pellet of the described composition.

For reusability tests, ABTS assays were repeated as mentioned above with the reused pellets. Before measuring the activities, the pellets were washed for 10 min in KPi before the second and the third cycle. The assay was also carried out with a higher stirring speed (600 rpm), to investigate mass transfer limitations.

1.3.2 Determination of the protein content

100 µL enzyme solution in 50 mmol·L^{-1} KPi with a DF of 50 was added to 1 mL Bradford reagent. The sample was mixed thoroughly and incubated at room temperature for 2 min. The spectrophotometer was

blanked with 1 mL Bradford reagent, and absorbance was measured at 595 nm. The calculation of the protein content was carried out based on a calibration with bovine serum albumin as a standard.

1.3.3 Enzyme leaching of the immobilized PaDa-I

The absorbance of 100 µL washing liquid in 1 mL Bradford reagent was measured as described above. Activity assay from the washing liquid was performed via standard ABTS assay as described above. Additionally, the protein content of the washing liquid was determined with SDS-PAGE, too. Therefore, a reference system of different dilutions of PaDa-I (DF: 1; 5; 10; 15; 20; 50; 100) were prepared with 50 mmol·L^{-1} KPi. Ten µL of each sample was added to a 1.5 mL micro reaction tube and mixed with 20 µL Laemmli sample buffer 2x. The samples were heated at 95 °C for 10 minutes with a BTD Dry Block Heater for Microtubes (Grant Instruments Ltd, Cambridge, UK). Then, 10 µL of the samples and PageRulerTM prestained protein ladder were added on an SDS plate (SurePAGE, Bis-Tris, 10x8, GenScript Corporation). Electrophoresis was run by a constant voltage of 140 V for 60 min. Afterwards, the gel was removed and rinsed with deionized water. The gel was stained with Coomassie Brilliant Blue for 45 min on Sunflower Mini-Shaker (Grant-Bio PS-3D). Samples from 5 mL washing liquid of 10 enzyme-containing hydrogels were measured analogously.

1.4 Swelling Degree

The physical characteristics (mass, height, and diameter) of 3 blank and 3 hydrogels containing immobilized PaDa-I were measured before washing, after one day, and after at least two days of washing in 50 mmol·L^{-1} KPi at room temperature. Equilibrium swelling degree ($q_{m,\infty}$) was calculated after at least 48 h equilibration in the washing buffer according to:

$$q_{m,\infty} = \frac{m_t}{m_0} - 1$$

where m_0 is the initial mass of the pellets, and m_t is the mass at equilibrium.

1.5 Experiments in continuous flow

For pumping the solutions, AZURA P 4.1S HPLC pumps (Knauer, Berlin, Germany) were used. The outflow was connected to an AZURA Detector MWD 2.1 L (Knauer, Berlin, Germany) equipped with a semi-preparative Knauer pressure-proof flow cell cartridge with a path length of 3 mm and a total volume of 2 µL. After the detector unit, a back pressure regulator (BPR) of 100 psi was installed to ensure that no gassing out occurs within the flow cell cartridge. Capillaries with an inner diameter of 0.75 mm were used. T-pieces were used to combine different flow streams, if necessary. The pumps and the detector were controlled by ClarityChrom chromatography data system software (Knauer, Berlin, Germany).

The immobilized enzyme was packed in a Tricorn 5 chromatography column (borosilicate glass, 50 mm tube height, 5 mm inner diameter; Cytiva, Brønshøj, Denmark). Two types of packing were examined: a

random film and a pellet packing. For the random film packing, the column was coated with 300 µL PaDa-I-hydrogel mixture, irradiated for 3.5 min at 365 nm under constant rolling and incubated under white light for 1 h. The column height was set to 5 cm and then filled with 50 mmol·L^{-1} KPi connected to the flow system and washed for 30 min. For the pellet packing, the column was filled with 30 pieces of 10 µL PaDa-I-hydrogel pellets (equivalent to 300 µL hydrogel film coating and treated as mentioned above.

After the washing of the enzyme-hydrogel packing material, $ABTS^{\bullet+}$ was enzymatically produced in continuous flow. Therefore, ABTS (0.3 mmol·L^{-1}, pH 4.4, 0.5 mL·min^{-1}) was flushed through the tubing and the detector for ca. 10 min to equilibrate the system and to blank the detector to the ABTS signal in continuous operation. An enzymatic run with a total flow rate of 0.5 mL·min^{-1} was performed with individual flow rates of 0.496 mL·min^{-1} ABTS solution and 0.004 mL·min^{-1} 0.035 wt% H_2O_2. The oxidation of ABTS was continuously determined at 405 nm. For the correlation of the measured absorbance with the concentration of $ABTS^{\bullet+}$, the following equation was applied.[2]

$$c_{ABTS^{\bullet+}}\left(\frac{\text{mmol}}{\text{L}}\right) = x\ (\text{mAU}) * 9.61 * 10^{-5}\left(\frac{\text{mmol}}{\text{L} \cdot \text{mAU}}\right)$$

The total amount of produced $ABTS^{\bullet+}$ was obtained by integrating the curves (see also "Calculation of continuous metrics" below).

2 Additional figures

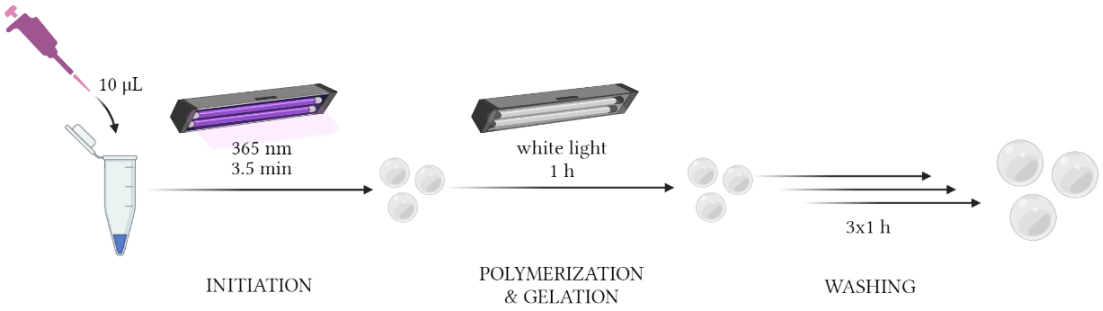


Figure S1. Schematic representation of the hydrogel-pellet preparation.

Figure S2. A pair of hydrogels before and after the washing in KPi. The photo shows an unwashed hydrogel pellet on the left and one after equilibration on the right.

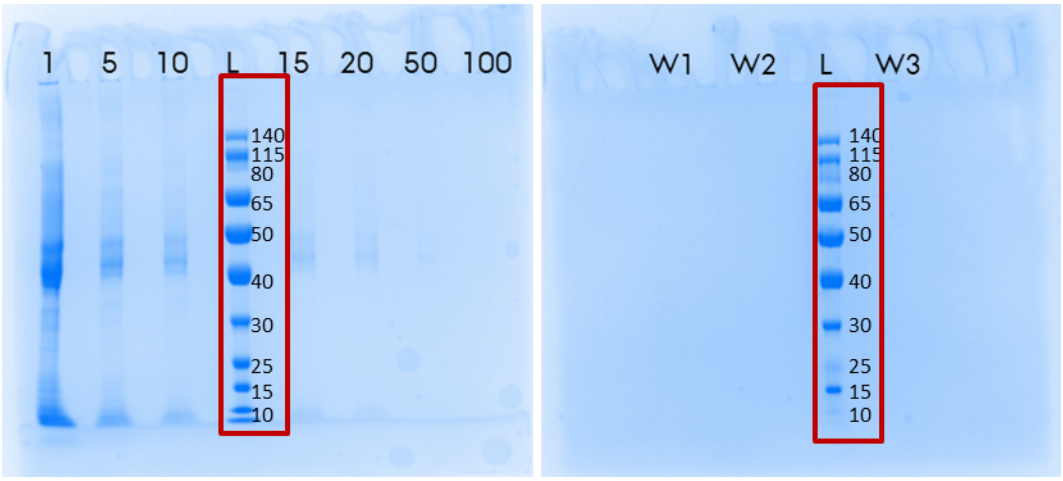


Figure S3. SDS-PAGE for the detection of protein content in the washing fractions. On the left: a reference system set up with different dilutions of the free enzyme. On the right: the three washing fractions of 10 hydrogels. Numbers indicate the dilution, as DF = 1, 5, 10, 15, 20, 50, and 100. L: PageRulerTM prestained protein ladder. W1, W2, and W3 are the first, second and third washing fractions, respectively.

3 Calculation of continuous metrics

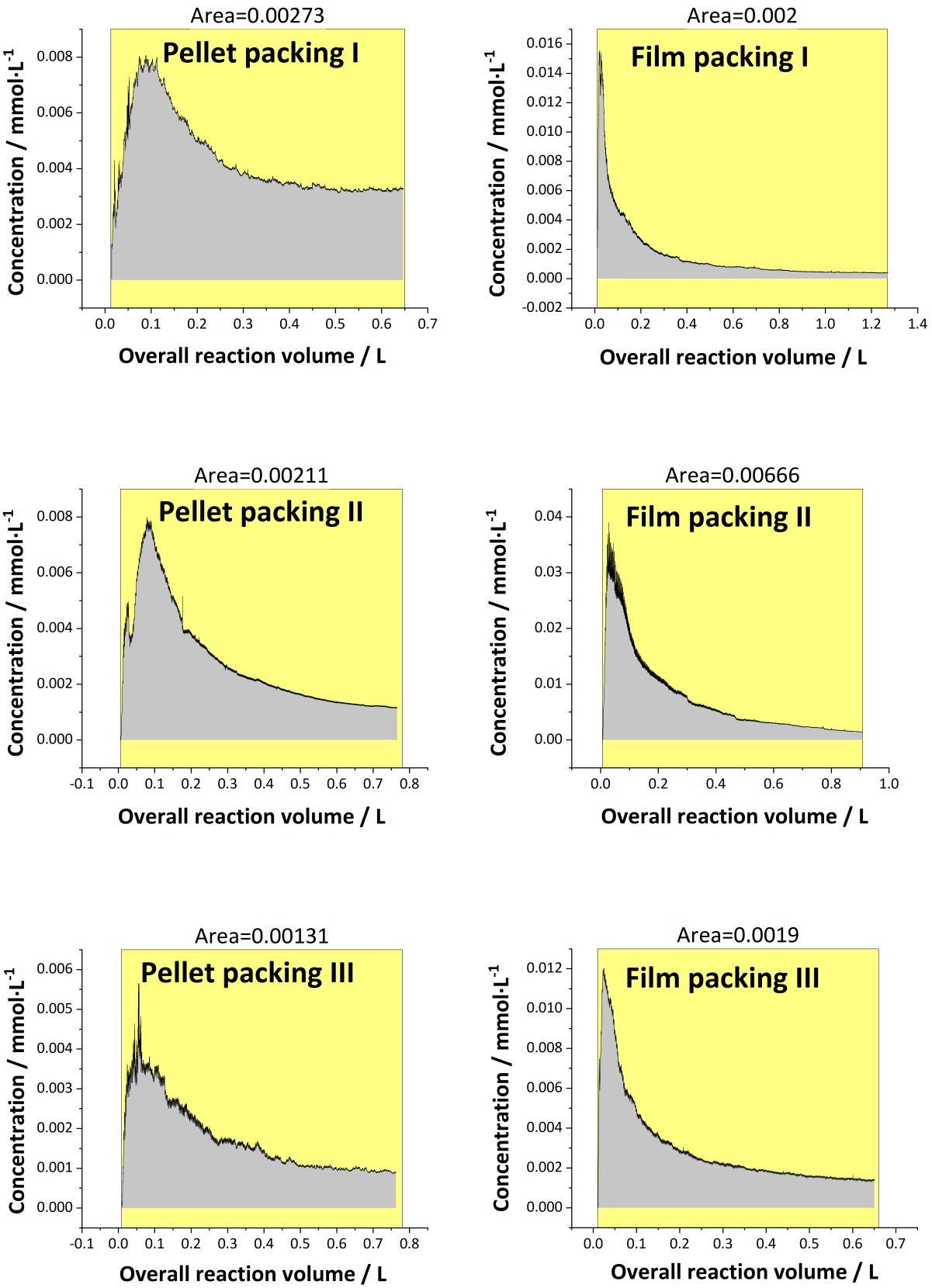


Figure S4. Integration results of the continuous biocatalytic flow experiments. Each graph represents a single experimental run. Experiments with pellet packing on the left and with film packing on the right.

Exemplarily calculation for the overall productivity and space time yield, based on the integration result for “film packing I”.

Production of ABTS$^{\bullet+}$, f = 0.5 mL·min^{-1} (= 0.03 L·h^{-1}); V_R: 3.93 mL

(1) $$n_{ABTS^{\bullet+}} = 0.002 \text{ mmol}$$

(2) $$c_{ABTS^{\bullet+}} = \frac{0.000002 \text{ mol}}{1.27 \text{ L}}$$

(3) $$c_{ABTS^{\bullet+}} = 1.57 * 10^{-6} \frac{\text{mol}}{\text{L}}$$

(4) $$m_{ABTS^{\bullet+}} = 0.000002 \text{ mol} \cdot 548.7 \frac{\text{g}}{\text{mol}}$$

(5) $$m_{ABTS^{\bullet+}} = 0.0010974 \text{ g}$$

(6) $$c_{ABTS^{\bullet+}} = \frac{0.0010974 \text{ g}}{1.27 \text{ L}}$$

(7) $$c_{ABTS^{\bullet+}} = 0.000861 \frac{\text{g}}{\text{L}}$$

STY, f = 0.5 mL·min^{-1} (= 0.03 L·h^{-1}); V_R: 3.93 mL; [P] = 0.000861 g·L^{-1}

$$\text{STY} = \frac{[\text{P}] \times f}{V_R}$$

$$\text{STY} = \frac{0.000861 \text{ g} \times 0.03 \text{ L}}{\text{L} \times \text{h} \times 0.00393 \text{ L}}$$

$$\text{STY} = 0.0066 \frac{\text{g}}{\text{L·h}}$$

4 Calculations of immobilization metrics

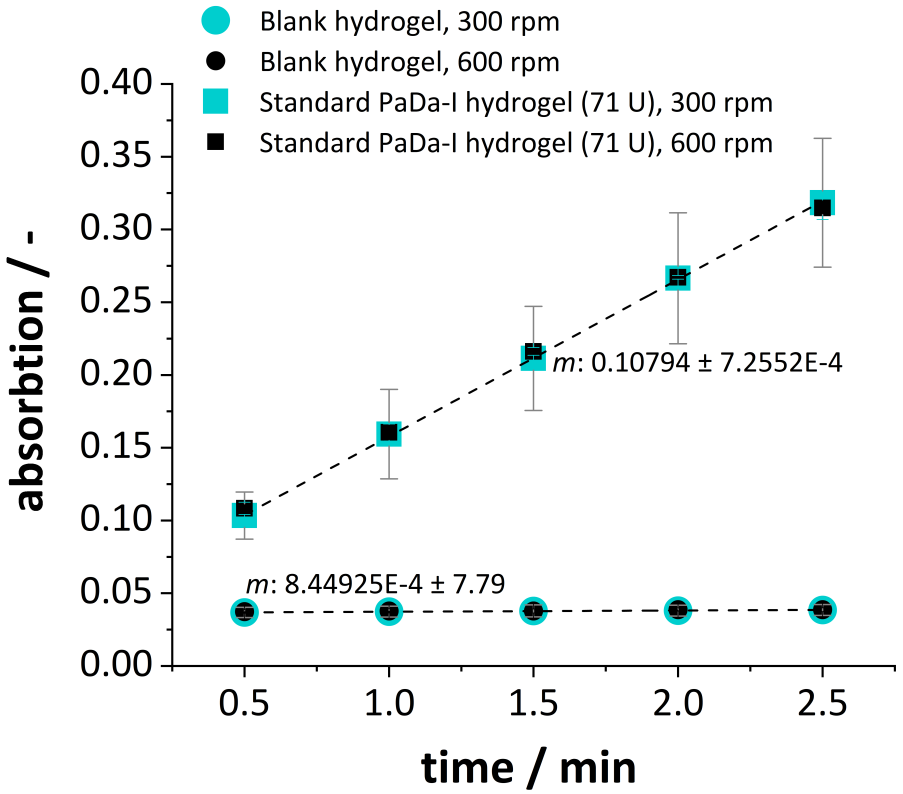


Figure S5. The activity of the immobilized PaDa-I at different stirring speeds. The average activity of triplicate measurements is compared to the "activity" of a blank hydrogel pellet without any enzyme. See also Table S1.

Example calculations for the activities of heterogenized enzymes (data from Figure S5, 300 rpm):

$$A_{\text{spec.}}\left(\frac{\text{U}}{\text{g}_{\text{wet carrier}}}\right) = \frac{\Delta\text{Abs}_{405} \cdot \text{DF} \cdot V}{\varepsilon^{\text{ABTS}^{\bullet+}}_{405\text{ nm}} \cdot m_{\text{wet carrier}} \cdot d} = \frac{0.10794\ \text{min}^{-1} \cdot 1 \cdot 0.01\ \text{L}}{36800\ \text{mol}^{-1}\ \text{cm}^{-1}\ \text{L} \cdot 0.0912\ \text{g} \cdot 1\ \text{cm}}$$

$$A_{\text{spec.}}\left(\frac{\text{U}}{\text{g}_{\text{wet carrier}}}\right) = 3.2 \cdot 10^{-7}\ \text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}_{\text{wet carrier}}$$

$$= 3.2 \cdot 10^{-1}\ \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}_{\text{wet carrier}}$$

$$= 3.2 \cdot 10^{-1}\ \text{U} \cdot \text{g}^{-1}_{\text{wet carrier}}$$

Table S1. Values of calculated activities. Compare also Figure S5.

Hydrogel	Activity / $U \cdot g_{wet\ carrier}^{-1}$
Blank hydrogel, 300 rpm	$2.4 \cdot 10^{-3}$
Blank hydrogel, 600 rpm	$2.0 \cdot 10^{-3}$
Standard PaDa-I hydrogel (71 U), 300 rpm	$3.2 \cdot 10^{-1}$
Standard PaDa-I hydrogel (71 U), 600 rpm	$3.1 \cdot 10^{-1}$

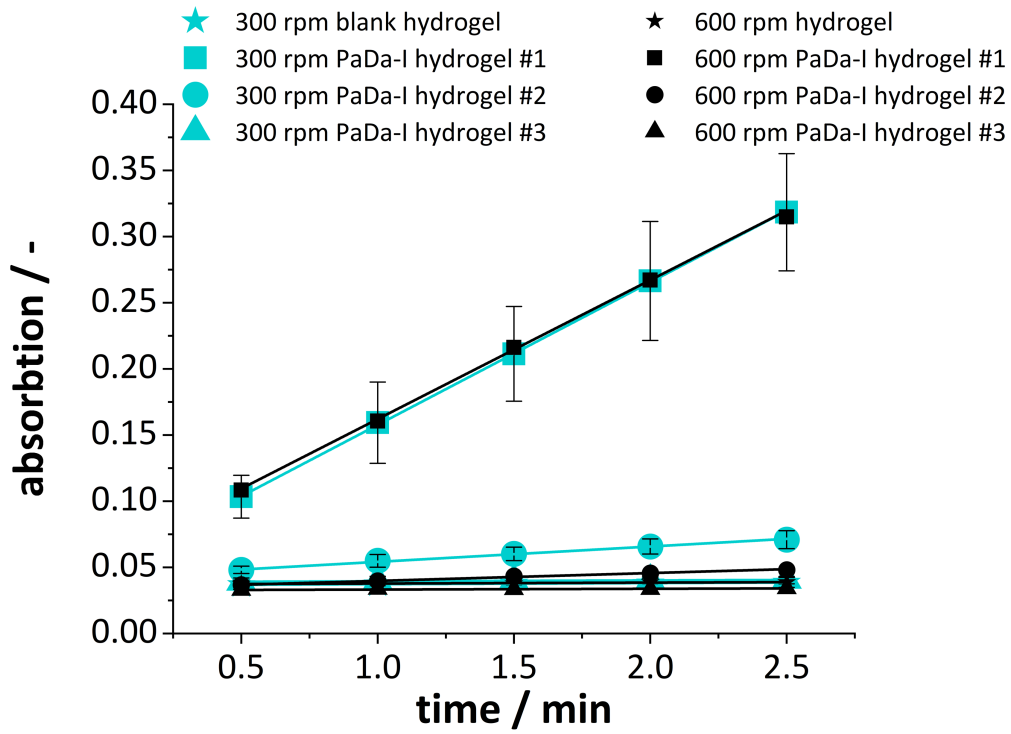


Figure S6. The reusability of hydrogels through three cycles for 300 rpm (cyan) and 600 rpm (black). Average activity is illustrated as result of triplicate measurements. Cycle numbers shown as #1, #2, and #3.

Table S2. Values of calculated activities. Compare also Figure S6.

Hydrogel	Activity / $U \cdot g_{wet\ carrier}^{-1}$
Blank hydrogel, 300 rpm	$2.4 \cdot 10^{-3}$
#1 Cycle PaDa-I hydrogel, 300 rpm	$3.2 \cdot 10^{-1}$
#2 Cycle PaDa-I hydrogel, 300 rpm	$4.3 \cdot 10^{-2}$
#3 Cycle PaDa-I hydrogel, 300 rpm	$2.8 \cdot 10^{-3}$
Blank hydrogel, 600 rpm	$2.0 \cdot 10^{-3}$
#1 Cycle PaDa-I hydrogel, 600 rpm	$3.1 \cdot 10^{-1}$
#2 Cycle PaDa-I hydrogel, 600 rpm	$2.3 \cdot 10^{-2}$
#3 Cycle PaDa-I hydrogel, 600 rpm	$2.3 \cdot 10^{-3}$

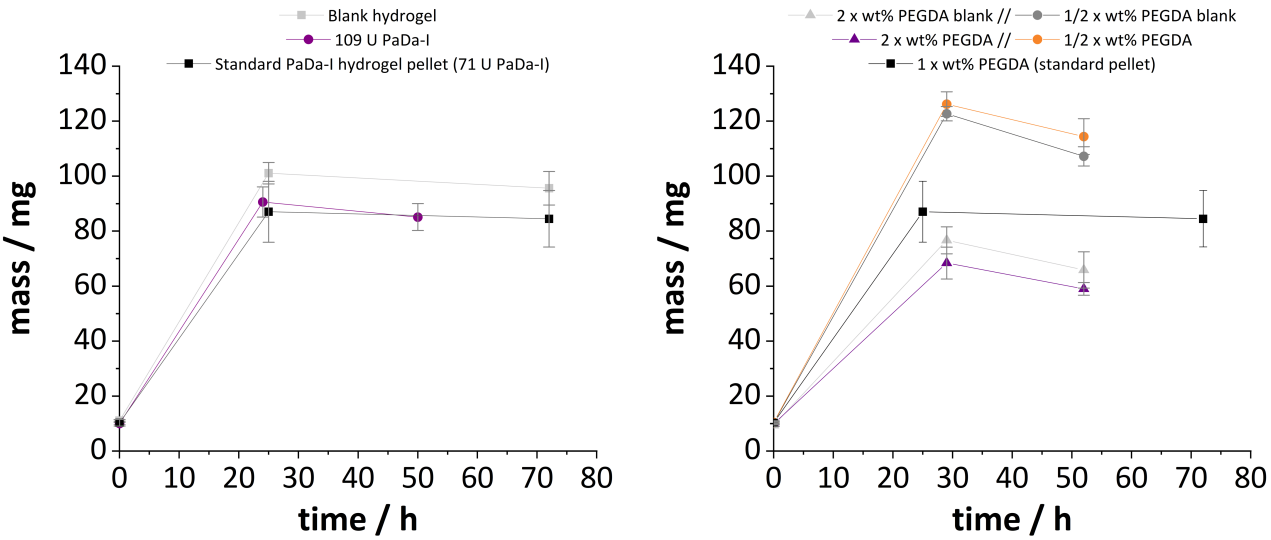


Figure S7. Swelling degrees of the hydrogel pellets under different conditions. A "blank hydrogel" (= hydrogel without enzyme) as well as a hydrogel pellet with higher enzyme amount ("109 U PaDa-I") show no significant difference in the swelling degree compared to a standard hydrogel pellet with 71 U of enzyme activity (left). By doubling the crosslinker amount PEGDA, a lower swelling degree was yielded ("2 wt% PEGDA blank"). Less crosslinker PEGDA amounts showed a higher swelling degree ("1/2 wt% PEGDA blank"). Both are compared to the standard pellet ("1 wt% PEGDA standard pellet", right). Values for $q_{m,\infty}$ see also Table S3.

Table S3. Values for the swelling degree. Compare also Figure S7.

Hydrogel	Swelling degree $q_{m,\infty}$ / -
109 U PaDa-I hydrogel	7.5
Standard PaDa-I hydrogel pellet (71 U)	7.2
1 x wt% PEGDA (standard pellet) = blank hydrogel	7.6
2 x wt% PEGDA blank	6.0
2 x wt% PEGDA	4.9
1/2 x wt% PEGDA blank	9.4
1/2 x wt% PEGDA	10.0

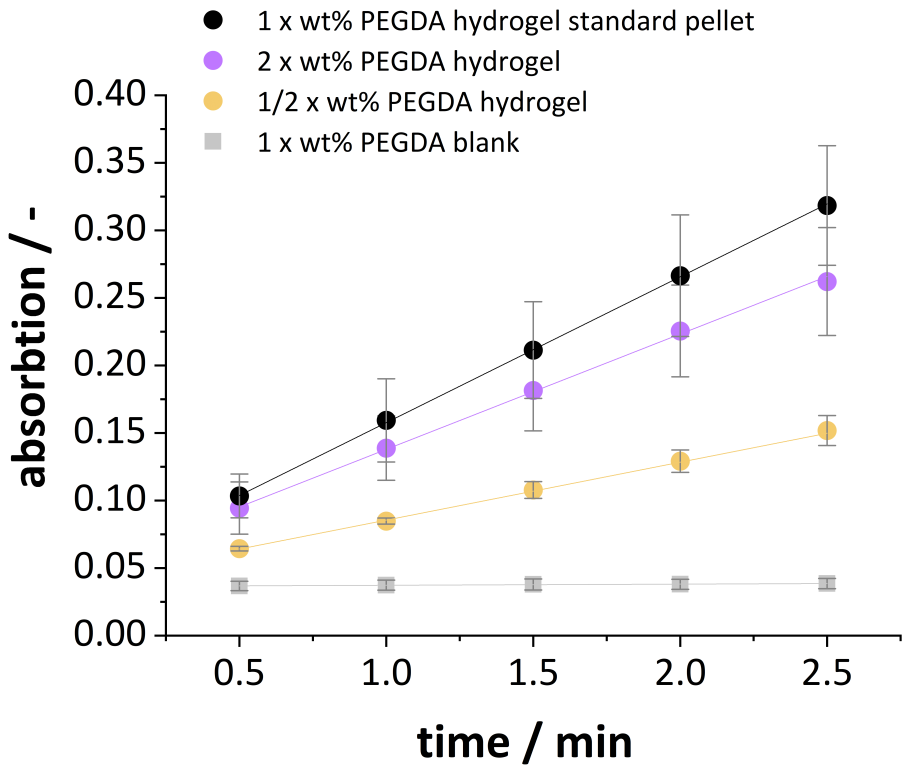


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Figure S8. Activity of pellets with altered amount of PEGDA 700 crosslinker compared with pellets containing half amount (1/2 wt%) of crosslinker or double (2 wt%). The graph shows results by 300 rpm agitation.

Table S4. Values for the activity with altered amount of PEGDA 700 crosslinker. Compare also Figure S8.

Hydrogel	Activity / $U \cdot g_{wet\ carrier}^{-1}$
1 x wt% PEGDA hydrogel standard pellet, 300 rpm	$3.2 \cdot 10^{-1}$
2 x wt% PEGDA hydrogel, 300 rpm	$3.6 \cdot 10^{-1}$
1/2 x wt% PEGDA hydrogel, 300 rpm	$1.1 \cdot 10^{-1}$
1 x wt% PEGDA blank, 300 rpm	$2.4 \cdot 10^{-3}$
1 x wt% PEGDA hydrogel standard pellet, 600 rpm	$3.1 \cdot 10^{-1}$
2 x wt% PEGDA hydrogel, 600 rpm	$3.5 \cdot 10^{-1}$
1/2 x wt% PEGDA hydrogel, 600 rpm	$1.2 \cdot 10^{-1}$

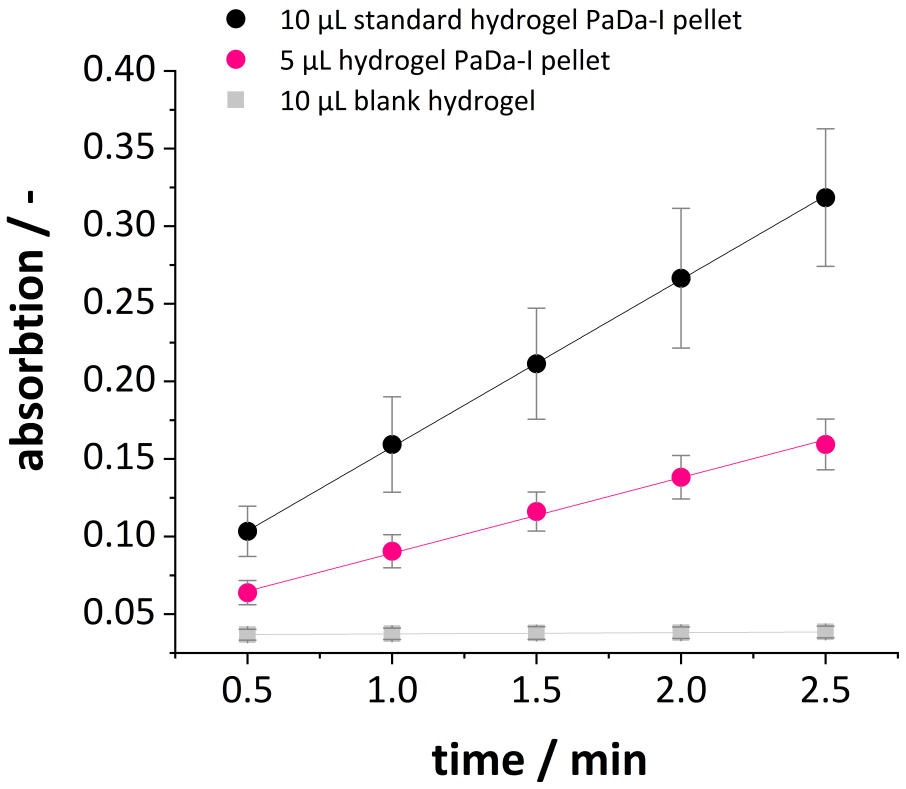

Figure S9. Comparison of the activity of a hydrogel with a Volume of 10 µL (= standard hydrogel pellets) and 5 µL prior to polymerization.

Table S5. Values for the swelling degree. Compare also Figure S9.

Hydrogel	Activity / $U \cdot g_{wet\ carrier}^{-1}$
10 µL standard hydrogel PaDa-I pellet	$3.2 \cdot 10^{-1}$
5 µL hydrogel PaDa-I pellet	$3.1 \cdot 10^{-1}$
10 µL blank hydrogel	$2.4 \cdot 10^{-3}$

5 References

1 M. Hobisch and S. Kara, *Automated Feeding and Fermentation Phase Transitions for the Production of Unspecific Peroxygenase by Pichia pastoris*, 2021.

2 L.-E. Meyer, B. Fogtmann Hauge, T. Müller Kvorning, P. De Santis and S. Kara, Continuous oxyfunctionalizations catalyzed by unspecific peroxygenase, *Catal. Sci. Technol.*, 2022, **12**, 6473–6485.