Electronic Supplementary Information

Hydrogel/enzyme dots as adaptable tool for non-compartmentalized multi-enzymatic reactions in microfluidic devices

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1. Equipment

X-ray Photoelectron Spectroscopy (XPS)

All XPS studies were carried out by means of an Axis Ultra photoelectron spectrometer (Kratos Analytical, Manchester, UK). The spectrometer was equipped with a monochromatic Al Kα ($h \cdot \nu$ = 1486.6 eV) X-ray source of 300 W at 15 kV. The kinetic energy of photoelectrons was determined with hemispheric analyzer set to pass energy of 160 eV for wide-scan spectra and 20 eV for high-resolution spectra. During all measurements, electrostatic charging of the sample was avoided by means of a low-energy electron source working in combination with a magnetic immersion lens. Later, all recorded peaks were shifted by the same value that was necessary to set the C 1s peak to 285.00 eV.

Quantitative elemental compositions were determined from peak areas using experimentally determined sensitivity factors and the spectrometer transmission function. Spectrum background was subtracted according to Shirley. The high-resolution spectra were deconvoluted by means of the Kratos spectra deconvolution software. Free parameters of component peaks were their binding energy (BE), height, full width at half maximum and the Gaussian-Lorentzian ratio.

Contact angle measurement (CA)

All contact angles were measured by sessile drop experiments as advancing (θ_a) and receding contact angles (θ_r). The advancing contact angle is the maximum contact angle which is determined upon increasing the droplet volume, whereas the receding contact angle is the minimum contact angle which can be measured by decreasing the droplet volume. All contact angle values were measured using an OCA 35xl drop shape analyzer (DataPhysics Instruments GmbH, Filderstadt, Germany). Droplets of de-ionized water (surface tension 72.8 mN/m at 23°C) were placed with a motor-driven syringe onto the sample surface. The syringe was kept in the droplet during the contact angle measurement. The software of the tangent method was used to calculate the corresponding contact angle. The contact angle values given here are mean values of three individual measurements carried out on different locations on the sample surface.

Ultraviolet–visible spectroscopy (UV-Vis)

Enzymatic activity tests were done measuring the absorption of ABTS at 405 nm with SPECORD® 210 PLUS (Analytic Jena AG, Jena, Germany) spectrometer using plastic cuvettes (Brand GmbH & Co KG, Wertheim, Germany). Data collection and evaluation was done with Win Aspect Plus (Analytic Jena AG, Jena, Germany).

UV-irradiation

Photopolymerization was performed using a light source with a wave length of 380 nm and an irradiation density of 8 W/cm² (DELOLUX 04, DELO Industrie Klebstoffe GmbH, Windach, Germany). Irradiation was done for 7.5 seconds with 8 cm space to sample to assure complete irradiation of the samples.

Microfluidic setup

For microfluidic tests the Asia syringe pump (Syrris Ltd., Royston, UK) with 50 and 100 µl syringes was used setting the flowrate from 5 µl/min until 250 µl/min. Detection was done using a Cary 50 spectrometer (Varian Inc., Palo Alto, CA, USA) with a 50 µl flow-through cell (Hellma GmbH, Müllheim, Germany) and the self-written program Cary Control.

Topographic analysis

Topographic analysis was done using the confocal 3D-microscope µsurf (NanoFocus, Oberhausen, Germany) visualizing the structured hydrogels on the glass surface at 25 °C and 50 % humidity. The recorded data analyzed by the device-specific software.

2. Surface activation and TPM-modification

Surface activation

Functionalized surfaces where cleaned and activated during an RCA-cleaning protocol. First the glass slides were treated with isopropanol, millipore-water and ethanol in an ultrasonic bath. The activation was done by slightly etching in H_2O_2 (33%)/ammonium hydroxide (28-30%)/millipore water (1:1:5) at 70 °C for 10 minutes followed by cleaning with millipore water and drying.

TPM-modification

3-(Trichlorosilyl)propyl methacrylate (TPM)-modification was performed by gas deposition in an argon-flushed desiccator at 20 mbar and room temperature within 2 hours. The functionalized glass slides were stored cooled and light protected for maximal two weeks. Analysis was done by XPS (Figure S1) and CA (Table ESI-1).

XPS measurements

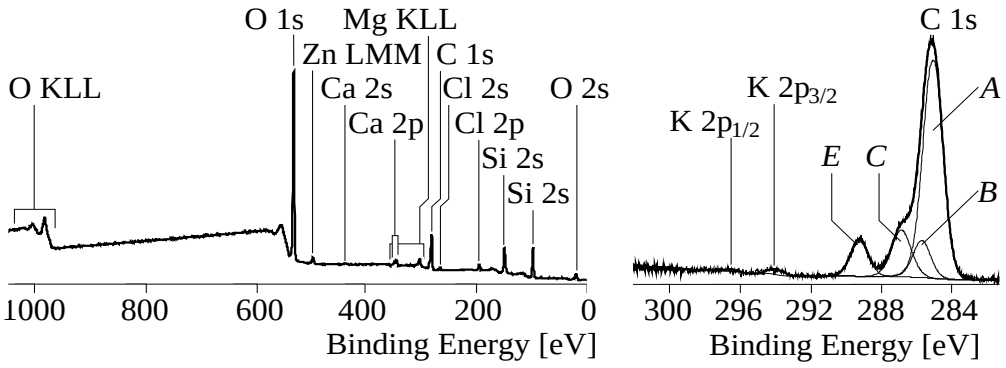

Figure ESI-1. Wide-scan and C 1s high-resolution XPS spectra of a TPM-functionalized glass sample.

The wide-scan spectrum (Figure S1, left) recorded from TPM-functionalized glass sample showed elements originated from the glass substrate (such as magnesium [the Mg 2p and Mg 2s peaks were observed at 50.8 eV and 89.6 eV] and calcium) and from the TPM layer, which was condensed on the silanol groups of the glass substrate surface. Both the glass and the silane contain silicon and oxygen. Assuming that all silicon is formally bonded as SiO_2 the elemental ratio would be [Si]:[O] = 0.5. Really, the [Si]:[O] ratio, which was determined from the wide-scan spectrum, was about 0.4. Obviously, the excess of silicon resulted from the presence of silyl moieties, which were condensed on the substrate surface. The shape of the C 1s high-resolution spectrum (Figure S1, right) is characteristic carboxylate ester. It was deconvoluted into four component peaks showing the differently bonded carbon atoms on the sample surface. The main component peak *A* (285.00 eV) showed the presence of saturated hydrocarbons. Electrons of carbon atoms of the allyl groups ($H_2\underline{C}=\underline{C}H–$) should have a slightly lowered binding energy. Since the chemical shift is expected to be smaller than 0.3 eV and considering the high intensity of component peak *A*, the separation of an additional component peak for the allyl-bonded carbon atoms was omitted. Carbon atoms, which were directly bonded to silicon (<u>C</u>–Si) also contributed to component peak *A*. The carbonyl carbons of the carboxylate ester groups (O=<u>C</u>–O–C) were identified as component peak *E* (289.18 eV). The photoelectrons escaped from the alcohol-sided carbon atoms of the ester groups (O=C–O–<u>C</u>) appeared as component peak *C* (286.86 eV). Carbon atoms in α-position to the carbonyl carbons (<u>C</u>–COOC) were found as component peak *B* (285.68 eV). As expected, the intensity of component peak *B* equaled the intensity of component peak *E*. Compared to component peak *E* the intensity component peak *C* was slightly increased. This indicates that small fraction of contaminations was adsorbed on the sample surface. The assumption is supported by the excess of saturated hydrocarbons, which were found on the sample surface.

Contact Angle Measurement

Table ESI-1. Results of the contact angle measurements carried out on the TPM-modified glass slides. For each value given here three individual measurements of the advancing (θ_a) and receding (θ_r) contact angle were averaged (δ means the statistical dispersion).

Sample	θ_a	δ	θ_r	δ
1	65.8	0.4	34.5	0.7
2	69.4	0.4	35.7	1.0
3	70.5	0.3	37.5	0.5
4	73.5	0.3	35.6	1.7
Ø	**70**	**3**	**36**	**1**

Comparative measurements on cleaned and activated glass surfaces were impossible because the water droplets immediately spread on the glass surface ($\theta_a < 5°$). The condensation of TPM on the glass surface is associated with the loss of numerous hydrophilic silanol surface groups. According to Table S1, the advancing contact angle values quantifying the wetting of the surface with water were about 70° ± 3°, and the corresponding receding contact angle values quantifying the dewetting of the surface were about 36° ±1°. The contact angle hysteresis of $\theta_a - \theta_r = 34°$ is mainly caused by the work of adhesion of the water molecules on the modified glass surface. Although the TPM modification of the glass surface resulted in significant increase in the contact angle values the modified surface can be considered as hydrophilic surface ($\theta_a < 90°$), which can be wet by aqueous solutions. The contribution of the surface-condensed propyl methacrylate layer to the partial hydrophobization excellently corresponded to the values given in literature.[1]

3. Synthesis of lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)

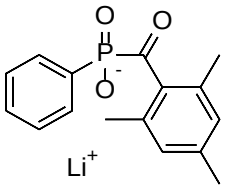

Figure ESI-2. Photoinitiator phenyl-2,4,6-trimethylbenzoylphosphinate.

NMR: ^{1}H NMR (500 MHz, D_2O, δ) [ppm]: 7.75 (t, J=8.99 Hz, Ph), 7.59 (t, J=7.30 Hz, Ph), 7.49 (t, J=1.00 Hz, Ph), 6.91 (s, Ph), 2.26 (s, Ph-C*H*$_3$), 2.05 (s, Ph-C*H*$_3$);

^{13}C NMR (500 MHz, D_2O, δ) [ppm]: 231.50 (d, *C*O-Ph), 142.75 (s, *Ph*-CH_3), 140.67 (d, *Ph*-CO), 136.57 (s, *Ph*-CH_3), 135.6 (d, Ph), 135.06 (d, Ph), 134.85 (d, Ph), 131.18 (d, Ph), 130.88 (s, Ph), 22.91 (s, Ph-*C*H_3), 21.30 (s, Ph-*C*H_3).

^{31}P NMR (500 MHz, D_2O, δ) [ppm]: 13.11 (s, Ph-*P*O_2-CO).

4. Synthesis and characterization of hydrogel dots

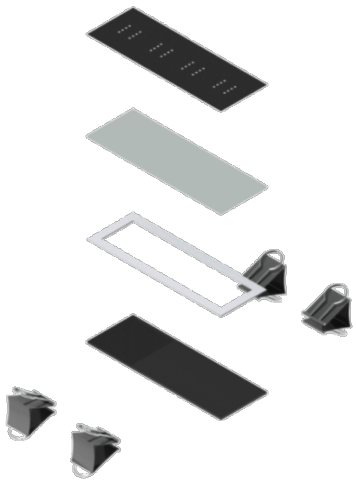

Figure ESI-3. Polymerization setup for preparation of structured hydrogel dots on glass surfaces including (top to bottom): mask, TPM-functionalized glass, adhesive tape, black PET-plate and clamps.

Figure ESI-4. Mask for polymerization of structured hydrogels on glass surfaces.

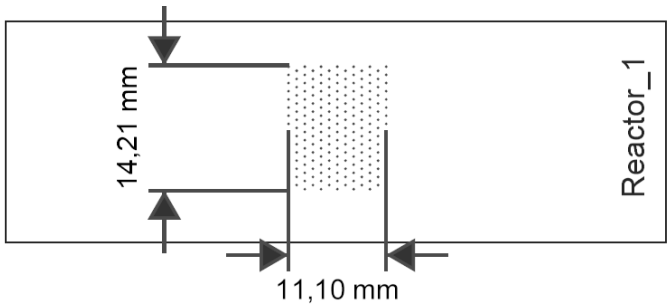

Figure ESI-5. Mask for polymerization of hexagonally organized hydrogel dots (Ø: 300 µm) on glass surfaces.

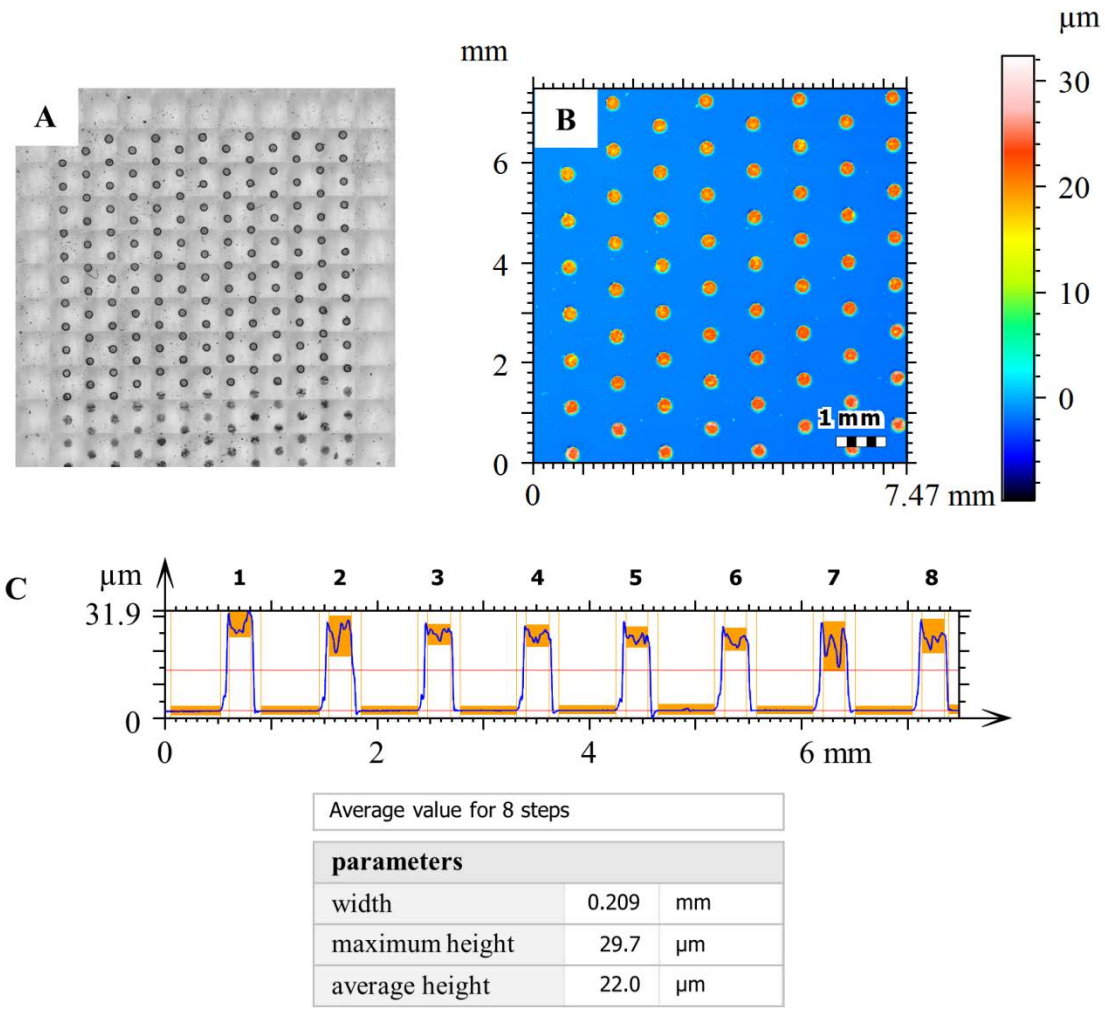

Figure ESI-6. µSurf measurement of hexagonally organized hydrogel dots, including their height determination, for microfluidic chamber reactor.

5. Calibration of ABTS Absorption within the microfluidic setup

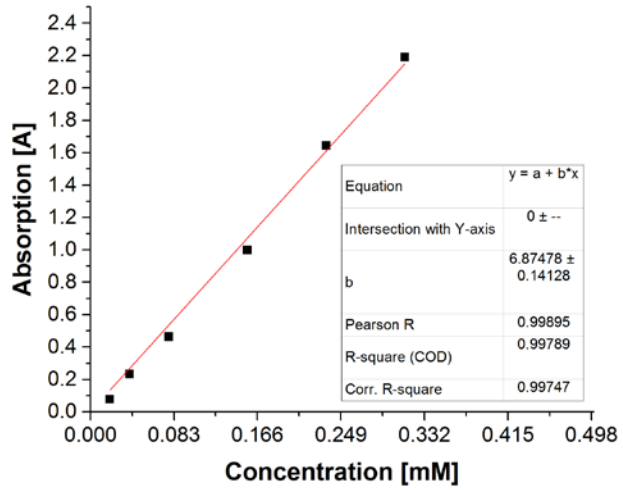

Figure ESI-7. Calibration curve of $[ABTS^{*}]^{+}$ for both tri-enzymatic cascade reactions.

6. Used stock solutions and isoelectric points of used enzymes

Stock solutions used in the study

Table ESI-2. Stock solutions for used ABTS and reaction educts for stability and long-term activity test of bi-enzymatic cascade reaction.

	ABTS	H_2O_2	D-Glucose	Lactose
Mass [mg]	109.7	204.1 µl (30% Solution)	360.3	342.3
V 0,1M PBS-buffer [ml]	2	1.8	2	2
c [mol/l]	0.1	1	1	0.5

Table ESI-3. Stock solutions for used buffer, ABTS, reaction educts and enzymes for stability and long-term activity test of tri-enzymatic cascade reaction.

Chemicals	Concentration (mol/l)	Enzymes	Concentration (mg/ml)
PBS-buffer	0.1	HRP	0.1
ABTS	0.1	GOx	0.05
H_2O_2	1	β-Gal	0.1
Glucose	1		
Lactose	0.5		

Table ESI-4. Enzyme concentrations in the hydrogel pre-solution for the patterning of hydrogel/enzyme dots on glass substrates.

	HRP	GOx	β-Gal
U/g solid	100000	228253	13400
c [mg/ml]	8.2	3.6	61.3

Table ESI-5. Stock solutions for used PBS buffer, ABTS and phosphatidylcholine (PC06) as educt for their use in microfluidic chamber reactor.

Chemicals	Concentration (mol/l)
PBS-buffer	0.1
ABTS	0.1
PC06	0,01

Isoelectric points of used enzymes:

HRP: 3.0-9.0 (Sigma Aldrich)

GOx: 4.2 (Sigma Aldrich)

β-Gal: 4.61 (Dashevsky[2])

ChOx: 4.1 ± 0.1 (Sigma Aldrich)

PLD (Streptomyces chromofuscus): 5,1[3]

7. Concentration depending activity measurement of free and immobilized enzymes

Concentration depending activity of immobilized enzymes for first tri-enzymatic cascade reaction

Concentration depending activity measurement of immobilized enzymes was done following the pipetting protocol shown in **Table ESI-6**. For the measurement the glass surface with 5 hydrogel rows was cut into 5 samples wearing each one row of hydrogel dots (20 hydrogel dots per row). Measurement was done over a period of 20 minutes measuring the absorption of $[ABTS^{*}]^{+}$ at

405 nm every 20 seconds. Activity was calculated from the determined linear absorption increase with the formula shown in equation S1.

Table ESI-6. Pipetting protocol of concentration depending activity of immobilized enzymes.

	Measurement HRP [µl]	Measurement GOx+HRP [µl]	Measurement β-Gal +GOx+HRP [µl]
ABTS	19.5	19.5	19.5
	39	39	39
	78	78	78
	117	117	117
PBS-buffer	2632.5	2632.5	2632.5
Substrate	H_2O_2	D-Glucose	Lactose
	19.5	19.5	19.5
	39	39	39
	78	78	78
	117	117	117

Equation S1: Calculation of enzymatic activity.

$$A = \frac{\frac{\Delta E}{\Delta t} \cdot V}{\varepsilon_\lambda \cdot d}$$

A	Activity [U]
ΔE/Δt	Linear absorption increase [1/min]
V	Measured volume [ml]
ε_λ	Molar extinction coefficient [L/(mmol·cm)] = 36.8 L/(mmol·cm) (λ=405 nm)
d	Passage length [cm] = 1 cm

Volume of HG-dots and enzyme content of hydrogel dot row

Table ESI-7. Volume determination of 20 hydrogel dots.

Diameter [µm]	Height [µm]	Volume per dot [nl]	Volume of 20 dots (1 hydrogel row) [nl]
250	20	0.9817	19.635

Table ESI-8. Amount of enzymes in one row of 20 hydrogel dots.

	HRP [ng]	GOx [ng]	β-Gal [ng]
Single-enzymatic	0.161	-	-
Bi-enzymatic	0.161	0.071	-
Tri-enzymatic	0.161	0.071	1.204

Concentration depending activity of free enzymes for first tri-enzymatic cascade reaction

Concentration depending activity measurement of free enzymes was done following the pipetting protocol shown in **Table ESI-9.** Following the calculation of the volume of one row of hydrogel dots (20 hydrogel dots) with a given enzyme concentration (Table ESI-8) the amount of free enzymes could be calculated and was used for activity measurement of free enzymes (Table ESI-8.). Measurement was done over a period of 20 minutes measuring the absorption of $[ABTS^{*}]^{+}$ at λ=405 nm every 20 seconds. Activity was calculated from the determined linear absorption increase with the formula shown in equation S1.

Table ESI-9. Pipetting protocol of concentration depending activity of free enzymes.

	Measurement HRP [µl]	Measurement GOx+HRP [µl]	Measurement β-Gal +GOx+HRP [µl]
ABTS	19.5	19.5	19.5
	39	39	39
	78	78	78
	117	117	117
HRP	3.2	3.2	3.2
GOx	-	2.77	2.77
β-Gal	-	-	23.6
PBS-buffer	2629.3	2611.1	2587.5
Substrate	H_2O_2	D-Glucose	Lactose
	19.5	19.5	19.5
	39	39	39
	78	78	78
	117	117	117

8. Long-term Activity – Measurement Settings for first tri-enzymatic cascade reaction

Following the same procedure as the activity measurement of immobilized enzymes a glass surface was cut into 5 samples and activity measurement was done with educt amount of 28 mM

(H_2O_2, glucose or lactose). Following the activity measurement the samples were stored in 3 ml PBS buffer at 8 °C.

9. Long term activity measurement of free and immobilized enzymes for second tri-enzymatic cascade reaction

Long-term activity measurement of the immobilized and free enzymes was done by preparing 500 µl enzyme-containing hydrogel solution (Table ESI-10.). This precursor solution was divided into two samples of 250 µl precursor solution. The photoinitiator LAP was added to one sample and out of this LAP-containing precursor the enzyme-containing hydrogel bulks were polymerized with 4 µl volume each bulk (immobilized enzymes). The other precursor sample was diluted with 750 µl PBS buffer (0.1 M) to prevent HRP-driven polymerization (free enzymes; known from literature).

Table ESI-10. 500 µl enzyme-containing hydrogel precursor solution for second tri-enzymatic cascade reaction. The LAP was added to one half of the solution (to 250 µl precursor solution).

	Mass [mg]	Volume [µl]
PBS buffer (0.1 M)	-	293,5
PEGDA	177,94	158,87
DMAEMA	-	18,04
HEMA	-	7,73
LAP	3,5 (into 250 precursor solution)	
Enzymes	Mass [mg]	Volume [µl]
HRP (100 U/mg)	4,1	-
ChOx (18,5 U/mg)	11,1	-
PLD (diluted to 9523 U/ml)	-	21,5

Long-term activity measurement of free and immobilized enzymes was done following the pipetting protocol shown in Table ESI-11. For each measurement one bulk hydrogel (4 µl volume, immobilized enzymes) or 16 µl non-polymerized and 4-fold diluted precursor solution (free enzymes) was placed in macro cuvettes. Measurement was done over a period of 40 minutes measuring the absorption of $[ABTS^{*}]^{+}$ at λ=405 nm every 30 seconds. Activity was calculated from the determined linear absorption increase with the formula shown in equation S1. Following the measurement the hydrogel bulks were stored in PBS buffer at 8°C. The non-polymerized precursor solution was stored light protected at 8°C to prevent polymerization.

Table ESI-11. Pipetting protocol for long-term activity measurement of free and immobilized enzymes for second tri-enzymatic cascade reaction.

	c [mol/l]	Volume [µl]
Enzyme-containing precursor	-	16 (free enzymes)
PBS buffer	0.1	1805 (immobilized enzymes) or 1789 (free enzymes)
ABTS	0.1	78
PC06	0.01	750

10. Activity of immobilized enzymes within hydrogels with different cationic charge for first tri-enzymatic reaction

Hydrogel pre-solution was prepared following the same procedure as described for the activity tests at different educt concentrations. Polymerization of hydrogel bulks was done in square-shaped polymerization molds (4x4x0.3 mm) each filled with 4 µl hydrogel pre-solution. Concentration of DMAEMA was varied from 0 % (40% HEMA) up to 40% (0% HEMA). The measurement was done over 20 minutes at 405 nm and absorption was measured every 20 seconds following the pipetting protocol shown in Table ESI-12. for one hydrogel bulk. Measurement was done 3 times for every measurement point. Activity was calculated using equation S1.

Table ESI-12. Pipetting protocol for activity measurement of enzymes within hydrogels containing different cationic charges for first tri-enzymatic cascade reaction.

	Concentration [M]	Volume [µl]
PBS buffer	0.1	2020
ABTS	0.1	390
Lactose	0.1	78

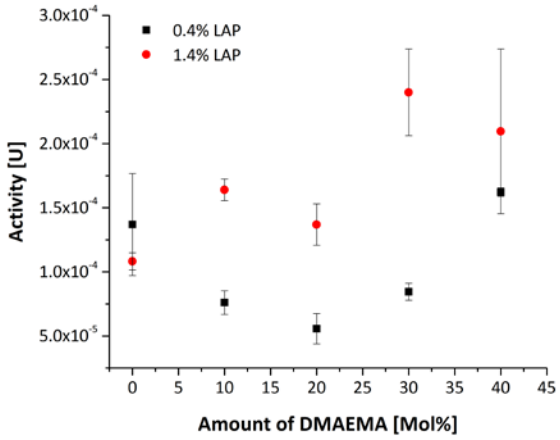

Figure ESI-8. Activity of first tri-enzymatic cascade reaction immobilized in hydrogels with varied amounts of DMAEMA.

11.Leakage test

Hydrogel pre-solution was prepared following the same procedure as described for the activity tests at different educt concentrations. Polymerization of hydrogel bulks was done in square-shaped polymerization molds (4x4x0.3 mm^3) each filled with 4 µl hydrogel pre-solution. 15 hydrogel bulks were produced and stored in 3 ml PBS buffer overnight. Initial activity was measured with enzyme containing non-polymerized hydrogel pre-solution following the pipetting protocol shown in Table ESI-13. Enzyme leakage was measured by activity measurement of storage solution and activity was compared to initial enzyme activity of hydrogel pre-solution. Activity was calculated using equation S1.

Table ESI-13. Pipetting protocol of leakage test for first tri-enzymatic cascade reaction.

	Concentration [M]	Volume [µl]
PBS buffer	0.1	1346.6
Storage solution / hydrogel pre-solution		673.3
ABTS	0.1	390
Lactose	0.1	78

12.Microfluidic Setup

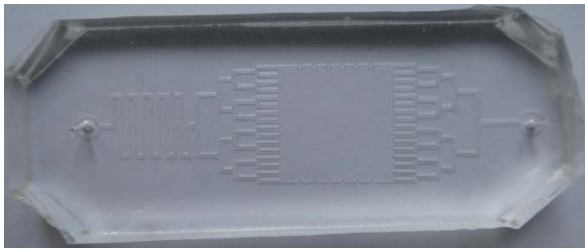

Figure ESI-9. Glass slide with hexagonally patterned hydrogel dots covered by the PDMS-mold with microfluidic chamber reactor.

Microfluidic Test under Flow Conditions

Microfluidic tests were done with 100 ml PBS buffer as fluid, containing 342.3 mg Lactose (0.01M) and 548.7 mg ABTS (0.01 M), placed in the depot of the syringe pump.

13.Simulation

Additionally simulation of different concepts of a reaction chamber has been done to prove different designs of a reaction chamber. Beside the square-shaped reaction chamber, a triangular reaction chamber has been simulated (Figure 9).

14.References

[1] W.G. Koh, A. Revzin, A. Simonian, T. Reeves, M. Pishko, Control of mammalian cell and bacteria adhesion on substrates micropatterned with poly(ethylene glycol) hydrogels, Biomed Microdevices, 5(2003) 11-9.

[2] A. Dashevsky, Protein loss by the microencapsulation of an enzyme (lactase) in alginate beads, International Journal of Pharmaceutics, 161(1998) 1-5.

[3] S. Imamura, Y. Horiuti, Enzymatic Determination of Phospholipase-D Activity with Choline Oxidase, J Biochem-Tokyo, 83(1978) 677-80.