Microfluidics featuring multilayered hydrogel assemblies enable real-time NMR-monitoring of enzyme cascade reactions

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Abstract

Compartmentalized chemical reactions at the microscale are interesting from many perspectives including (multi)functional surfaces and biotechnology. Monitoring the molecular content as a measure of functional performance at these small scales is challenging. As a means to address this challenge, we leverage microtechnology and biocompatible materials to integrate a compact, reconfigurable reaction cell featuring electrochemical functionality with high-resolution nuclear magnetic resonance spectroscopy (NMR). We demonstrate the operation of this system by monitoring the activity of enzymes immobilized in chemically distinct layers within a multi-layered chitosan hydrogel assembly. As a benchmark, we observed the parallel activities of urease (Urs), catalase (Cat), and glucose oxidase (GOx) by recording NMR spectra to extract reagent and product concentrations in real-time. As a result, simultaneous monitoring of a cooperative enzymatic process (GOx + Cat) together with an independent process (Urs) is achieved. Using Michaelis-Menten progress curve analysis of the NMR data, kinetic data is extracted: in the case of GOx, the Michaelis constants (K_M) are consistent with previous reports, while for Urs, deviations are observed, attributed to an inhibitory effect under our reaction conditions. The system therefore enables the construction of complex reaction cascades with spatial control, as would be interesting in, for example, metabolic engineering and multiplexed sensing applications.

Introduction

Reproducing metabolic reactions in a controlled space that mimics the native micro-environmental conditions inside biological matter is an endeavour that can have important repercussions on bio-medical applications^[1]. Spatial and temporal control over the processes that regulate metabolic activity allows to fine-tune the dynamics underlying the metabolic reaction cascade, thereby enabling researchers to engineer i) models that imitate biological environments at various spatial scales (i.e. extra- and intracellular spaces) and ii) metabolic systems with

an optimized spatial arrangement of specific catalysts for a targeted application (e.g. bio-waste management, bio-production). From a medical perspective, several diagnostic platforms have been developed to inspect biomolecular abundance of metabolites with high sensitivity^[2], allowing early detection of diseases^[3] and providing valuable insights into system-level^[4] chemistry.

Highly hydrated polymer networks (hydrogels) are commonly used to mimic and interface with biological spaces, and their sophistication continues to evolve to keep pace with the desired performance in drug delivery, tissue engineering, and biosensing applications.^[5–8] There are numerous possibilities to generate hydrogels and their composites, and one attractive route to achieve localized hydrogels in both space and time is to uti-

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lize the stimuli-responsive hydrogel sub-class. In this class, the sol-gel transition can be controlled and triggered by applying an appropriate signal; in some cases, the sol-gel transition is reversible making the system attractive, for example, in biosensing applications where the hydrogel is resettable dependent on the sensing demands. Electrochemical-based fabrication of chitosan hydrogels is one system exploited for hydrogel assembly with high spatio-temporal precision.^[9,10] Chitosan is an attractive material given its biocompatibility, reversible gelation, and amenability to chemical functionalization properties.^[11–14] In addition, electrodeposition is relatively uncomplicated, requiring a two electrode system in contact with a chitosan solution to generate a local increase in pH with an applied current, thereby causing local deposition of the chitosan hydrogel. This has been exploited for (bio)sensing^[15–18] and controlled release^[19,20] applications.

Since the deposition process is controlled by an electrical signal, the architecture of the hydrogel can be varied simply by modulating the trigger signal. This results in a multilayered structure,^[21,22] which can be used to encode information^[23] or to generate cell bioreactors.^[24] Taking advantage of the ability to chemically functionalize chitosan prior to deposition, each layer can be additionally assigned a unique chemical identity.^[25] In this way, complex hydrogel structures can be assembled with programmable spatial separation of chemical function. Given that control of layer thickness can be on the order of micrometers, there is potential to leverage this technology in the direction of replicating the spatial localization of metabolic and functional elements within tissues. At these length scales, microfluidic methods are critical for the handling and management of the resultant small sample volumes.

Nuclear magnetic resonance (NMR) spectroscopy in combination with microfluidics has been identified as a powerful analytical method when both small sample management and *in situ* investigation of metabolic activity are required.^[26–28] NMR is non-invasive and reports on the dynamics of a (bio)chemical reaction with high resolution along both time and frequency (i.e. chemical) dimensions. In the context of NMR monitoring of localized metabolic activity, the challenge is to integrate additional functionalities into the detection space without degrading the SNR of the NMR signal and without perturbing

the homogeneity of the local magnetic environment necessary for high quality NMR spectra. It was previously reported that integration of hardware elements for simple electrochemical manipulations inside the active region of an NMR micro-detector is possible, without compromising the overall quality of the recorded spectrum^[29].

In this report, we describe functional monitoring of spatially localized enzymes within a microfluidic device by a micro-NMR system. By electrodeposition, a chitosan hydrogel was co-localized with an electrode while the catalytic potential perpendicular to the electrode surface was controlled by sequential deposition of chitosan layers modified with different enzymes. Urease (Urs), glucose oxidase (GOx), and catalase (Cat) were selected for a three-layer system, representing independent conversion of urea (to ammonia via Urs) and glucose (to gluconic acid via GOx) and a coupled enzymatic reaction (peroxide generated by GOx to water via Cat). In the microfluidic device, conversion of approximately 3 μmol of urea and 0.3 μmol of glucose by a multi-functional chitosan hydrogel was measured, demonstrating simultaneous monitoring of multiple biocatalytic reactions within a hydrogel matrix.

Results and Discussion

Platform configuration

A custom NMR probe head insert, compatible with a commercial Bruker Micro5 microimaging NMR probe, was designed and fabricated to enable NMR monitoring of an *in situ* electrodeposition experiment (Figure 1). The NMR detector used in this work is a Helmholtz pair with a 1.2 mm diameter and 600 μm separation between the two windings.^[27] The microcoil was mounted on top of a PCB, hosting both the ^1H NMR channel tracks path, interfaced to the NMR probe head, and chitosan electrodeposition tracks, connected to a power source outside of the magnet using a PCB pin-header (Figure 1a). The support structure of the probe insert was fabricated in PMMA.

Two variations of an application-specific sample insert^[27] were considered for the *in situ* electrochemistry experiment. For the first variation, the electrode pair was planar and was patterned on the bottom surface of the microfluidic channel (planar configuration): one round

working electrode with a 1.5 mm diameter (total area 1.76 mm²) and a rectangular counter electrode 1.5 mm × 2 mm. In this case the microfluidic channel had a total sample capacity of 3.2 µL.

For the second variation, the electrodes were placed along the walls of a 1.1 mm-wide fluidic channel (side-wall configuration) so that, when inserted into the NMR detector, the electrodes were at the periphery of the Helmholtz coil. In this configuration, the area of both the working and the counter electrodes is 0.43 mm² and the total sample capacity is 2.6 µL. Details regarding the design and fabrication of the electrodeposition sample inserts, as well as a discussion about the compatibility with the Helmholtz NMR detector, have been reported elsewhere.^[29]

Electrical connection to the host PCB electrodeposition lines was done by soldering tin-plated copper wire to the gold pads of the microfluidic insert (Figure 1c). Fluidic contact with the insert was achieved by interfacing soft silicon tubing to the glass insert inlet and outlet ports using custom connectors (machined Teflon) and soft PDMS pads (Figure 1b). The silicon tubing was fed to the top of the insert assembly and connected to fluidic lines outside of the magnet bore.

After assembly and installation of the probe, a typical NMR experiment proceeded as follows (Figure 1c): 1) a solution containing chitosan functionalized with the desired enzyme was flushed through the system to fill the microfluidic insert; 2) a DC current was applied to the pair of electrodes for 15 min to perform hydrogel electrodeposition; 3) a rinsing solution (buffered at pH 7) was gently flushed through to stabilize the hydrogel; 4) a solution containing the desired substrates was flushed through the system. Depending on the particular experiment, steps 1-3 were repeated to build a layered hydrogel before flushing the gel stack with substrate solution. After step 4) the probe was inserted into the magnet bore to begin the NMR measurement routine.

NMR spectra were collected on a Bruker AVANCE system operating at a ¹H Larmor frequency of 500.13 MHz. A series of spectra were collected, each acquisition requiring 9 min with a 15 min delay between subsequent acquisitions, so that initiation of acquisitions were separated by 24 min in time. The microfluidic insert placement was such that the working electrode in the planar configuration was *not* within the NMR detector (i.e. the hydrogel

Figure 1: Experimental setup for in situ electrodeposition of pre- and post- modified chitosan inside the microfluidic chip. a) overview of the NMR probe head insert used in this work, showing the main elements of the device: a1) PCB hosting RF tracks, tuning and matching fixed capacitors, the electrodeposition tracks and the NMR microcoil, a2) the microfluidic insert mounted inside the Helmholtz pair microcoil, a3) insert base with RF female connectors, a4) microfluidic connectors and connectors clamps, a5) topside connector supporting both electrical and fluidic connections to the outside. b) schematic of fluidic components, left side view and top view. c) highlighted electrical components: c1) electrodeposition lines and PCB header connector, c2) tin-plated copper connections between PCB and microfluidic insert, c3) microcoil NMR detector, c4) ¹H NMR RF lines. d) schematic illustration of the *in situ* electrodeposition process: d1) loading the microfluidic channel with the polymer solution, d2) applying a DC bias to the electrode pair to initiate electrodeposition, d3) rinsing with a buffer and/or loading the channel with a reagent solution.

was not in the detector), while the electrodes were placed at the periphery of the detector in the side-wall configuration (i.e. the hydrogel was in the detector).

Preparation of enzymes bound to CSPEG

In this work, urease (Urs: jack bean, EC 3.5.1.5), catalase (Cat: bovine liver, EC 1.11.1.6), and glucose oxidase (GOx: from *Aspergillus niger*, EC 1.1.3.4) were chosen as model enzymes to verify that i) covalent enzyme attachment to chitosan does not inhibit their catalytic ability; ii) catalytic activity is maintained when deposited as a multi-layered hydrogel; iii) microfluidic NMR can be used to monitor catalytic turnover. Both Urs and GOx have been observed to be catalytically active while integrated within a hydrogel.^[30]

The enzymes were connected to chitosan using a di-functional polyethylene glycol (PEG) linker and NHS/EDC coupling chemistry. It was observed previously that PEG retains molecular mobility when tethered to chitosan,^[29] and therefore it was anticipated this mobility would be useful in maintaining enzyme function. Coupling efficiency to the PEG-modified chitosan was estimated by UV/Vis spectroscopic analysis of the reaction solution after precipitation of the hydrogel (Figure S2). The coupling efficiencies observed were 41% for Urs and 35% for GOx (Table 1). This value is important when determining the number of active units of enzyme as describe below.

Monitoring enzyme activity

The number of active enzyme units extracted from the initial slopes of the kinetic data was used to determine the catalytic performance under various conditions. These conditions included enzyme in solution, enzyme-coupled polymer in solution, and enzyme-coupled polymer in the hydrogel state.

(1) *Enzymes in solution, pre- and post-coupling.* The number of active units was used as a metric to investigate the influence of the coupling chemistry and hydrogel immobilization on enzyme performance. Urs was tested by transferring a solution containing 2 mg mL^{-1} Urs + 1000 mM urea to the microfluidic channel ($3.2\text{ }\mu\text{L}$) for NMR measurement (pH 5.5, NaAc). The resulting NMR

data are plotted in Figure 2a (pink). As per the manufacturer data sheet, $0.633 \pm 0.006\text{ U}$ of Urs were expected using 2 mg mL^{-1} Urs, while the measurement revealed $0.057 \pm 0.013\text{ U}$ of Urs, i.e. the active fraction was $9 \pm 2\%$.

The procedure was repeated using CS-Urs in 1000 mM urea solution (pH 5.5, NaAc). The results are presented in Figure 2a (pink). The CS-Urs solution was prepared so that the concentration of Urs was 2 mg mL^{-1} and, accounting for the coupling yield, $0.259 \pm 0.007\text{ U}$ of Urs was expected. The measurement revealed $0.076 \pm 0.025\text{ U}$ of Urs were present, i.e. $29.3 \pm 9.8\%$ (data summarized in Table 1).

The fraction of active Urs units was observed to be higher for CS-Urs in solution compared to free Urs in solution ($29.3 \pm 9.8\%$ vs. $9 \pm 2\%$). One would expect that the restricted diffusion together with deactivation of a fraction of the enzymes due to the coupling chemistry would decrease the efficiency.^[31,32] This counter intuitive result may be the result of the increased buffering effect of the CS polymer, maintaining better control of pH during the reaction as compared to the case of Urs in solution. In the absence of CS, the pH was observed to increase from 5.5 to 9 at the end of the reaction, while the presence of CS controlled the pH such that it increased from 5.5 to 6.5 (Figure S5a). At elevated pH, Urs activity is expected to be limited as it has been reported that Urs maintains activity from below pH 3 to 7.5.^[33,34]

The GOx / Cat system was similarly evaluated in the microfluidic system, starting with a solution containing 4 mg mL^{-1} GOx and 1 mg mL^{-1} Cat + 100 mM glucose (pH 5.5, NaAc). Figure 3a plots the NMR data, in this case both reactant consumption (glucose) and product formation (D-gluconic acid) could be followed. From the data sheet, $1.265 \pm 0.013\text{ U}$ GOx were anticipated, and from the NMR data $2.9 \pm 0.1\text{ mU}$ were observed ($0.23 \pm 0.01\%$ active fraction).

To perform the CS-coupled experiments, two polymer solutions were prepared: CS-GOx and CS-Cat. The two polymer solutions were mixed to maintain the 4:1 ratio of GOx:Cat, and maintain the expected concentration of GOx at 4 mg mL^{-1} . Finally, the polymer solution was added to a glucose solution (100 mM) before transfer to the microfluidic system and NMR measurement (results in Figure 3b (top), spectra in SI, Figure S9 (a and b)). The active units were determined to be $2.5 \pm 0.6\text{ mU}$,

Figure 2: (a) Representative ^1H NMR spectra obtained for the electrodeposited CS-Urs hydrogel. The hydrolysis of urea (highlighted in pink, 5.8 ppm) is shown as a function of time, and (b) extracted urea concentration in the presence of: top - Urs (open symbols) and CS-Urs (filled symbols) in solution; bottom - immobilized CS-Urs. The urea concentration was calculated using the internal standard (TSP, 50 mM). The fits to the data were obtained using the Lambert W function (Equations 1 and 2), kinetic parameters summarized in Table 1. Results for Urs and CS-Urs in solution are plotted in Figure S8.

which was 0.49 ± 0.12 % of the 519 ± 14 mU added (after accounting for coupling yield) (data summarized in Table 1).

The fraction of active GOx was observed to be significantly less as compared to Urs, and only up to approximately 70-80 % of the initial glucose was consumed. The solution pH cannot explain this observation: the optimal pH for GOx activity is in the range 4.0-7.5^[35,36] which was maintained in our system (Figure S5b). A possible explanation for the incomplete substrate conversion is an oxygen deficiency. In the presence of catalase, 0.5 mol of oxygen is consumed for every mol of glucose. Since the reaction volume was only $3.2 \mu\text{L}$ and the materials used to fabricate the device restrict oxygen diffusion into the solution, oxygen could have become the limiting reagent as the reaction progressed. This is supported by control experiments performed in standard 5 mm NMR tubes, where the sample volume is $500 \mu\text{L}$ and a large gas headspace is available in the NMR tube. Every other parameter held constant, a factor of 2-4 improvement in the fraction of active units was observed (details in Table S3). This highlights the importance of considering oxygen perfusion in microfluidic devices in

cases where it participates in the process.^[37]

(2) *Enzymes immobilized in chitosan hydrogel.* To verify the enzymes retained their activity post-electrodeposition, hydrogels were generated using the same CS-enzyme solutions as were functionally verified in solution. For CS-Urs, the microfluidic device was filled with a 1 % w/v solution and 3 A m^{-2} of current was applied for 15 min to the electrodes within the channel. Once electrodeposition was complete, the fluidic channel was gently flushed with 200 mM NaAc and D_2O to stabilize the hydrogel before introducing a 1000 mM urea solution (in 200 mM NaAc, pH 7) to initiate the reaction. For NMR measurements, the sample insert was placed so that the planar electrode did not enter the NMR detection volume. The resulting NMR spectra (one time point every 24 min) and extracted concentrations are plotted in 2a and b (bottom).

The NMR measurement revealed $0.53 \pm 0.16 \mu\text{U}$ of Urs were present in the system. To estimate the fraction of Urs that was active, it was first necessary to estimate the volume of the hydrogel (details in Supporting Information). Given the dimensions of the planar electrode (circle with radius 0.6 mm) and a deposition time

of 15 min yielding a thickness of $\sim 15 \mu\text{m}$,^[25] a volume of $26.5 \pm 0.3 \text{ nL}$ could be estimated. Given the coupling yield was 41 %, the fraction of Urs that was active was then $24.4 \pm 7.4 \%$ (summarized in Table 1).

In the case of the GOx and Cat, a two layer hydrogel was generated (multi-layered hydrogels will be denoted as layer-1 / layer-2 / layer-3). Using the same polymer concentration and electrodeposition parameters as for CS-Urs, the two layer hydrogel was built by depositing CS-GOx, rinsing and stabilization with 200 mM NaAc buffer in D_2O , then deposition of CS-Cat, followed by rinsing and stabilization so that a CS-GOx / CS-Cat hydrogel was created. To initiate the reaction, 200 mM glucose was flushed into the microfluidic channel (in 200 mM NaAc, pH 7) and NMR measurements were started. The NMR spectra and extracted concentrations (glucose at 5.2 ppm, gluconic acid at 4.0, 4.2 ppm) are plotted in Figure 3a and b (bottom).

As in the case of the Urs hydrogel, GOx was observed to retain its activity when coupled to chitosan and electrodeposited as a hydrogel, as has been reported.^[38] The number of active units were determined to be $23 \pm 3 \text{ nU}$, with a fractional activity of $0.51 \pm 0.06 \%$ (summarized in Table 1).

Comparison between the chitosan-coupled cases reveals that the activity of both Urs and GOx is not influenced after deposition as a hydrogel. This result is not completely surprising since by definition the hydrogel is highly hydrated, and thus diffusion of the substrate through the polymer network should be minimally restricted.

Multiplexed sensing of enzymatic activity

Enzyme functionality is maintained after coupling and deposition as a hydrogel. Catalytic cascades also remain active, even when catalytic steps are spatially separated as in the dual-layer CS-GOx / CS-Cat example. The multi-layer hydrogel assembly process was further extended to include multiple, independent catalytic reactions. A three layer hydrogel was deposited with the composition CS-Urs / CS-Cat / CS-GOx (schematic Figure 4a). This multiplexed system was prepared using two different electrode geometries, taking advantage of our recent investigation into optimizing electrode placement with respect to the NMR detection volume.^[29] The planar geometry, as was

used for CS-Urs and CS-GOx / CS-Cat examples, was used to place the catalytic hydrogel assembly slightly upstream (i.e. outside) of the NMR detector. The side-wall electrode geometry, with better NMR performance, was used to place the hydrogel within the NMR detector.

The hydrogel was assembled using the same protocol as described above - for each layer a sequence of CS-modified polymer solution injection into the microfluidic device, electrodeposition for 15 min, and rinsing (using 200 mM NaAc, pH 7). The final assembly was then exposed to a solution containing substrates for both Urs and GOx: 1000 mM urea and 100 mM glucose (200 mM NaAc, pH 7). The ^1H NMR spectra and extracted concentration profiles versus time are presented in Figure 4 for both electrode geometries.

The NMR data clearly show the ability to monitor both reactions (urea hydrolysis, glucose oxidation) simultaneously. The substrates (urea, glucose) and product (gluconic acid) all have unique signals that can be used for quantitative determination of the catalytic processes. The extracted kinetic data is summarized in Table 2. Accounting for the expected number of active units within the respective hydrogel layers, the fraction of active enzymes were determined to be essentially independent of hydrogel assembly and electrode geometry (Table 2). A summary of these results is presented in Figure 5.

Enzyme kinetics

Urease. The initial slope (V_{max}) for all Urs measurements were observed to be identical (within our experimental error): $13 \pm 3 \text{ mMmin}^{-1}$ for the single layer of CS-Urs, $14.0 \pm 4 \text{ mMmin}^{-1}$ for CS-Urs in solution, $15.0 \pm 3 \text{ mMmin}^{-1}$ for multi-layer CS-Urs, and $15.0 \pm 7 \text{ mMmin}^{-1}$ for multiplex CS-enzymes in solution (Table 1). Conversely, the extracted K_M value was observed to be dependent on the Urs chemical environment. For a single layer hydrogel of CS-Urs, K_M was observed to be $540 \pm 120 \text{ mM}$, while for CS-Urs solution, the K_M value was $800 \pm 130 \text{ mM}$. In the multiplex system, the difference in CS-Urs K_M values was less extreme, $400 \pm 120 \text{ mM}$ for the hydrogel assembly and $360 \pm 100 \text{ mM}$ for the multiplexed CS-enzyme in solution (summary of K_M ranking: $\text{CS-Urs}_{\text{solution}} > \text{CS-Urs}_{\text{gel}} > \text{CS-Urs}_{\text{multiplex, gel}} > \text{CS-Urs}_{\text{multiplex, solution}}$).

Figure 3: (a) Representative ^1H NMR spectra obtained for the electrodeposited CS-GOx* / CS-Cat hydrogel. Concentrations were determined using the internal reference (TSP, 50 mM). The depletion of glucose (marked red, 5.8 ppm) upon conversion to gluconic acid (marked blue, 4–4.2 ppm) is shown as a function of time, and (b) extracted glucose and gluconic acid concentrations in the presence of: top - GOx and Cat in solution (open) and CS-GOx, CS-Cat in solution (filled); bottom - electrodeposited CS-GOx / CS-Cat. Best-fit curves obtained using Equations 1 and 2 are also shown. The resulting kinetic parameters are summarized in Table 1. *GOx experiments were always performed using GOx:Cat in a 4:1 ratio. Results for GOx and CS-GOx in solution are plotted in Figure S9.

The value of K_M extracted from these data are approximately 2 orders of magnitude larger than what is reported for jack bean urease (typically 1–4 mM).^[39] For Urs, K_M has insignificant variability as a function of pH,^[34,40] temperature,^[34,41] and pressure.^[41] Interpreting K_M in terms of binding affinity, these results suggest that the Urs immobilization significantly influences substrate binding affinity. Immobilization has been observed to affect urease function,^[42,43] the degree of influence strongly dependent on the immobilization substrate, chemistry, and measurement conditions.^[44] Here, we additionally observe K_M 1000 ± 230 mM for Urs free in solution (i.e. also uncharacteristically high). As discussed by Stroberg and co-workers,^[45] reliable kinetic data can be extracted when i) initial substrate concentration is on the order of K_M , and ii) initial enzyme concentration is less than K_M . Under our reaction conditions, $[\text{urea}]_{\text{initial}} \gg K_M$ while the initial enzyme concentration is certainly less than K_M , casting uncertainty on the extracted values. Inhibition is likely an additional contribution to the observed increase in K_M values. Inhibition constants for urea (substrate)

and ammonium (product) are in the range 3–6.4 M and 2–118 mM,^[39] within an order of magnitude of the initial urea concentrations used in these experiments (1 M). This concentration was selected in order to ensure an observable NMR signal, and could be reduced to a value closer to K_M and alleviate inhibition to verify this hypothesis, but these experiments were outside the scope of this work.

Glucose oxidase. As in the case of the Urs, the extracted V_{max} values for GOx was observed to be essentially independent of experimental conditions (note, in all GOx experiments, Cat was maintained at a ratio of 4:1): $0.85 \pm 0.08 \text{ mMmin}^{-1}$ for the single layer CS-GOx, $0.87 \pm 0.04 \text{ mMmin}^{-1}$ for CS-GOx in solution, $0.9 \pm 0.1 \text{ mMmin}^{-1}$ for multi-layered CS-GOx, and $0.9 \pm 0.3 \text{ mMmin}^{-1}$ for multiplex CS-enzymes in solution (Table 1). In contrast to the Urs experiments, K_M variation was less pronounced for GOx. For the CS-GOx / CS-Cat hydrogel, K_M was observed to be 60 ± 10 mM while for CS-GOx + CS-Cat in solution the value was 80 ± 20 mM. In the multiplex system, K_M was 22 ± 4 mM

Experiment		$V_{\max}$	K_M	Container Volume	Coupling Efficiency	Theoretical Units	Active Units	Active Fraction	
		mM/ min	mM	nl	-	mU	mU	%	
Urs	Solution	Urs + urea	11 ± 3	1000 ± 230	3160 ± 30	-	633 ± 6	60 ± 10	9 ± 2
		CS-Urs + urea	14 ± 4	800 ± 130	3160 ± 30	0.41 ± 0.01	259 ± 7	80 ± 30	30 ± 10
		Urs / GOx* + urea, glu- cose	14 ± 3	500 ± 160	3160 ± 30	-	633 ± 6	90 ± 10	14 ± 2
		CS-Urs / CS-GOx* + urea, glucose	15 ± 7	360 ± 100	3160 ± 30	0.41 ± 0.01	259 ± 7	90 ± 20	34 ± 7
	Gel	CS-Urs + urea	13 ± 3	540 ± 120	26.5 ± 0.3	0.41 ± 0.01	2.17 ± 0.06	0.5 ± 0.2	24 ± 7
		CS-Urs / CS-GOx* + urea, glucose	15 ± 3	400 ± 120	22.6 ± 0.2	0.41 ± 0.01	1.85 ± 0.05	0.63 ± 0.09	34 ± 5
GOx	Solution	GOx + glucose	0.98 ± 0.04	280 ± 70	3160 ± 30	-	1270 ± 10	2.9 ± 0.1	0.23 ± 0.01
		CS-GOx + glucose	0.87 ± 0.04	80 ± 20	3160 ± 30	0.35 ± 0.01	440 ± 10	2.5 ± 0.6	0.6 ± 0.1
		Urs / GOx* + glucose, urea	1.5 ± 0.5	38 ± 6	3160 ± 30	-	1270 ± 10	5 ± 3	0.4 ± 0.2
		CS-Urs / CS-GOx* + glucose, urea	0.9 ± 0.3	25 ± 5	3160 ± 30	0.35 ± 0.01	440 ± 10	2.7 ± 0.2	0.60 ± 0.04
	Gel	CS-GOx* + glucose	0.85 ± 0.08	60 ± 10	26.5 ± 0.3	0.35 ± 0.01	3.7 ± 0.1	0.023 ± 0.003	0.60 ± 0.07
		CS-Urs / CS-GOx* + glucose, urea	0.9 ± 0.1	22 ± 4	26.5 ± 0.3	0.35 ± 0.01	3.7 ± 0.1	0.024 ± 0.005	0.6 ± 0.1

Table 1: Summary of the kinetic activity of **Urease** (Urs) and **Glucose Oxidase** (GOx) using the microfluidic system. Substrate solutions contained either 1000 mM urea, 100 mM glucose (or a mixture thereof), and 50 mM TSP in 200 mM NaAc, pH 7 buffer. Kinetic parameters were extracted by fitting the consumption of urea and the production of gluconic acid, respectively. A comparison of GOx kinetic parameters using glucose consumption is given in Table S4. *Note: The GOx reactions were always prepared maintaining a 4:1 ratio with Cat.

and 25 ± 5 mM for the hydrogel and solution cases, respectively. These values compare well with reported values (33 – 110 mM),^[46,47] suggesting native activity is retained in the immobilized state.

Impact of hydrogel geometry. The extracted kinetic parameters for the two electrode geometries are summarized in Table 2. The $V_{\max}$ values for both Urs and GOx were independent of geometry; however, K_M for Urs was significantly different while for GOx, K_M was geometry insensitive. As observed in Figure 4, the new feature in the data was the incomplete conversion of urea in the side-wall electrode case. After calculating the number of active Urs units (Table 2), a significant difference between the two geometries is noted (0.63 ± 0.09 mU vs. 0.12 ± 0.03 mU for planar and side wall), and thus the ratio of substrate (urea) to active units was a factor of 5 larger for the side wall case. As a result, we speculate that the inhibitory effect of both the substrate and product was therefore stronger.

Conclusion

The compositionally customizable microfluidic system described in this study reveals the dynamics of immobilized multiplexed enzymes within an NMR detection volume. The kinetic data for the enzymes Urs and GOx obtained at 303 K are consistent with Michaelis-Menten kinetics (Equations 1 and 2) at pH 7.0. Each immobilized enzyme (Urs and GOx) performs its selective chemistry independent of the substrate mixture (urea and glucose), as expected. By spatially separating the functionality in the layered hydrogel assembly, new dimensions to studying enzymatic processes are envisioned, for example: (i) by controlling layer thicknesses and chemical identity, mass transit between layers can be tuned; (ii) multi-step chemical conversion can be designed by controlling the spatial location of the relevant catalysts; (iii) more than one independent reaction cascade can be programmed into the hydrogel assembly, enabling multiplexed experiments. In this work, biological catalysis was demonstrated, and this could be extended to homogeneous and heterogeneous catalytic processes in situa-

Figure 4: (left side) Illustration of the enzymatic reactions performed with hydrogel immobilized Urs, GOx, and Cat. Position of NMR-integrated hydrogens are highlighted in red (glucose), blue (gluconic acid), and pink (urea). The arrangement of the hydrogel assembly is displayed for the case of a planar electrode (top) and a sidewall electrode (bottom). Gel layers have been colored in blue for CS-GOx, grey for CS-Cat, and pink for CS-Urs. (a and b) Representative ^1H NMR time series of Urs and GOx enzymatic reactions. Experiments were performed on (a) a planar electrode and (b) a sidewall electrode. Signals are colored according to the color coding in the left schematic (urea at 5.8 ppm, the glucose signal at 5.3 ppm, the gluconic acid signal at 4.2 ppm). (c and d) Concentration time series of reactants and products extracted from NMR spectra in (a) and (b), by integrating NMR signals in the highlighted regions. Left axis: concentration of urea; right axis: concentrations of both glucose and gluconic acid. Solid lines are best-fit curves to the Michaelis-Menten kinetic model (Equations 1 and 2). Kinetic data are summarized in Table 2. Reproducibility is presented in Figure S7. Data for the enzyme mixture in solution, free and CS-coupled, are presented in Figures S10 and S11.

tions where the chemistry is water based (taking advantage of the hydrogel architecture) and the catalyst can be immobilized (chemically or physically) to distinct hydrogel layers. Cases in which the presence of one catalyst is deleterious to the function of another catalyst (e.g. by binding interference) can be additionally considered given the ease of compartmentalization enabled by the layered assembly. Sophisticated multi-functional surfaces can therefore be designed, applicable (non-exclusively) to metabolic engineering, (bio)sensing, and biomimicry investigations.

Experimental Section

Theoretical basis for progress curve analysis

Data analysis. NMR data were processed using the TopSpin (Operating and processing software for NMR spectrometers), Matlab2016b and Origin 2016.

The quality and reproducibility of the NMR-based approach to reaction kinetics monitoring depends largely on how reliably the NMR peak area integrals can be converted to concentrations. Here the peak area integrals from recorded ^1H -NMR spectra were converted into concentration values using the known concentration of TSP

Figure 5: Summary and comparison of the fraction of active enzyme units calculated under the three chemical environments, as well as using two different NMR detectors: standard 5 mm sample tube, 5 mm saddle coil; microfluidic system described in this report. Three different subcategories of experiments were grouped, i.e. single enzyme (one enzyme), multiplex (mixed enzymes) in solution, and electrodeposited (ED) multilayered assembly.

added to the sample ($[TSP] = 50 \text{ mM}$). Determining the molecular concentration using this approach reduces the uncertainties in the quantification of peak areas, since each sample has its own internal TSP reference and allows a comparison of data collected from different experiments. In those cases where the relevant signal overlapped with a larger one (e.g. glucose signals overlapping with the water signal) a non-linear baseline correction was applied before performing signal integration.

The initial slope of the concentration profile $S(t)$, i.e. the reaction velocity at the very beginning of the reaction, was estimated by calculating the numerical derivative of the best fit curve to the Michaelis-Menten model at $S(t) = 5 \text{ min}$. The initial concentration parameter S_0 was allowed

a $\pm 5\%$ uncertainty over the nominal initial concentration since there were not enough data points near $S(t) = 0$ to allow accurate extrapolation of the intercept when this parameter is allowed to run freely. The reaction velocity extracted from reagent concentration curves and expressed in mM/min was converted to $\mu\text{mol/min}$ using the known volume of either solution or gel containing the enzyme. This operation allows to estimate the absolute amount of product produced per minute, and therefore estimate the units of active enzymes. By definition, a *unit* (U) of enzyme is the amount required to produce $1 \mu\text{mol}$ of product per minute, under optimal reaction conditions.

Additional reaction kinetics parameters can be extracted from the time-dependent concentration $S(t)$ by us-

Experiment	$V_{\max}$ mM/min	K_M mM	Container Volume nl	Coupling Efficiency -	Theoretical Units mU	Active Units mU	Active Fraction %
Planar Electrode							
CS-Urs / CS-GOx*	15 ± 3	400 ± 120	22.6 ± 0.2	0.41 ± 0.01	1.9 ± 0.1	0.63 ± 0.09	34 ± 5
CS-Urs / CS- GOx *	0.9 ± 0.1	22 ± 4	26.5 ± 0.3	0.35 ± 0.01	3.7 ± 0.1	0.02 ± 0.01	0.6 ± 0.1
Sidewall Electrode							
CS-Urs / CS-GOx*	12 ± 2	130 ± 80	6.1 ± 0.3	0.41 ± 0.01	0.50 ± 0.01	0.12 ± 0.03	24 ± 5
CS-Urs / CS- GOx *	1.0 ± 0.1	23 ± 8	10.8 ± 0.5	0.35 ± 0.01	1.52 ± 0.05	$(8.0 \pm 0.5) \times 10^{-3}$	0.54 ± 0.04

Table 2: Summary of the kinetic activity of the multi-layered enzymes using two different electrode configurations, planar and sidewall electrodes. Substrate solution composition was 1000 mM urea, 100 mM glucose (200 mM NaAc, pH 7 + 50 mM TSP) in each case. Each row reports the extracted data for the enzyme highlighted in **bold**. *Note: GOx is always prepared together with Cat in a 4:1 ratio.

ing the well established Michaelis-Menten enzyme kinetics model.

For a reagent time series the following equation holds for the time evolution of the concentration $S_r(t)$ of a reactant^[48]:

$$S_r(t) = S_\infty + K_M W \left[\frac{S_\infty - S_0}{K_M} e^{\frac{(S_\infty - S_0) - V_{\max}(t-t_0)}{K_M}} \right] \quad (1)$$

For product concentration $S_p(t)$:

$$S_p(t) = S_\infty - K_M W \left[\frac{S_\infty - S_0}{K_M} e^{\frac{(S_\infty - S_0) - V_{\max}(t-t_0)}{K_M}} \right] \quad (2)$$

where S_0 is the concentration of reagent at time t_0 , S_∞ is the concentration at the conclusion of the reaction, K_M (Michaelis constant) corresponds to the reagent concentration at which half of the enzyme's active sites are occupied, $V_{\max}$ is the maximum reaction velocity. W is the Lambert W-function, the inverse function of $f(W) = W \exp W$. Details about the derivation of Equations 1 and 2 are reported elsewhere.^[45]

The descriptions of microfluidic fabrication, preparation of the samples and processes for the electrodeposition of chitosan, enzyme coupling and yield quantification, and NMR characterization are available in the Supporting Information.

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Keywords

chitosan, urease, glucose oxidase, catalase, conjugation, multiplex, hydrogels, NMR

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Supplementary Information

Microfluidics featuring multilayered hydrogel assemblies enable real-time NMR-monitoring of enzyme cascade reactions

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Compartmentalized chemical reactions at the microscale are interesting from many perspectives including (multi)functional surfaces and biotechnology. Monitoring the molecular content as a measure of functional performance at these small scales is challenging. As a means to address this challenge, we leverage microtechnology and biocompatible materials to integrate a compact, reconfigurable reaction cell featuring electrochemical functionality with high-resolution nuclear magnetic resonance spectroscopy (NMR). We demonstrate the operation of this system by monitoring the activity of enzymes immobilized in chemically distinct layers within a multi-layered chitosan hydrogel assembly. As a benchmark, we observed the parallel activities of urease (Urs), catalase (Cat), and glucose oxidase (GOx) by recording NMR spectra to extract reagent and product concentrations in real-time. As a result, simultaneous monitoring of a cooperative enzymatic process (GOx + Cat) together with an independent process (Urs) is achieved. Using Michaelis-Menten progress curve analysis of the NMR data, kinetic data is extracted: in the case of GOx, the Michaelis constants (K_M) are consistent with previous reports, while for Urs, deviations are observed, attributed to an inhibitory effect under our reaction conditions. The system therefore enables the construction of complex reaction cascades with spatial control, as would be interesting in, for example, metabolic engineering and multiplexed sensing applications.

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1 Supporting Information 1: Materials and Procedures

1.1 Materials

Enzymes: Urease (from *Canavalia ensiformis* (Jack bean)) with a specific activity of 100,000 U/(g of enzyme) (pH 7.6, 303 K, EC 3.5.1.5, MW~544,620 Da), glucose oxidase (from *Aspergillus niger*) with a specific activity of $\geq 100,000$ U/(g of enzyme) (type X-S, pH 7.6, 303 K, EC 1.1.3.4, MW~160,000 Da) and catalase (from bovine liver) with a specific activity of lyophilized powder, 2,000-5,000 U/mg protein (EC 1.11.1.6) were purchased from Sigma-Aldrich.

Substrates: Urea (powder, BioReagent, for molecular biology, suitable for cell culture), D-glucose ($\geq 99.5\%$ (GC)) and glucono- δ -lactone ($\geq 99.0\%$) were purchased from Sigma-Aldrich.

NMR solvents: Deuterium oxide D₂O (99.9 atom % D), and 3-(trimethylsilyl)- propionic-2,2,3,3-d₄ acid sodium salt (99.9 atom % D, contains 0.05 wt. % TSP), deuterium chloride (99 atom % D DCl), and deuterium sodium oxide (99 atom % NaOD), were purchased from Sigma-Aldrich.

Polymers: chitosan from crab shells (85% deacetylation, 200kDa), poly(ethylene glycol) bis(carboxymethyl) ether (average MW= 250, PEG), were purchased from Sigma-Aldrich.

Coupling reagents: N-(3-(dimethylamino)propyl)-N-ethylcarbodiimide hydrochloride (EDC) 99%, N-hydroxy-succinimide (NHS) 97% were purchased from Sigma-Aldrich.

Buffers: 200 mM of sodium acetate (NaAc) was made with NaAc tablets in deuterated (D₂O) water. The pH was adjusted with deuterium chloride (DCl), and deuterium sodium hydroxide (NaOD). All enzyme and modified-CS solutions and dilutions used this stock buffer to maintain the pH 5.5 (for solution) and pH 7 (for hydrogel) and the same concentration of TSP (50 mM).

1.2 Electrode microfabrication

The fabrication process was based on previous work with minor modifications.^[29] Briefly, fabrication started with a 210 μ m thick, 4-inch diameter glass wafer. The substrate was cleaned using a sequence of acetone, isopropanol (IPA),

and deionized water (DI) baths. An O₂ plasma treatment helped to improve wetting of the glass surface by polar photoresists during spin coating. The wafer was further dry-baked on a hotplate at 200 °C for 5 min. After baking, the substrate was sputtered with two different seed layers of 1/2 nm chromium/gold (Cr/Au) thickness. The substrate was cleaned again using a sequence of acetone, IPA, and DI baths for 10 min followed by an O₂ plasma treatment.

To define the counter-electrode, SU-8 3025 epoxy-based negative photoresist (UV-exposed regions become cross-linked, unexposed film is washed during development) was coated at 4000 rpm for 60 sec, and underwent soft-baking for 12 min at 95 °C. The dried photoresist was exposed using an i-line mask aligner (MA6, Karl Süss, Germany) with a dose of 350 mJ cm⁻² at 365 nm under hard contact, followed by post exposure bake at 95 °C for 4 min. Development was done using PGMEA developer for 2 min to achieve the desired undercut. The wafer was washed thoroughly with a sequence of coarse PGMEA, fine PGMEA, and IPA each for 10 min, followed by rinsing using DI water. The substrate was dry baked overnight.

The substrate was then electroplated with Au for 40 min to achieve a target thickness of 2 µm. SU-8 was stripped using a microwave plasma etching. A secondary SU-8 layer was spincoated and patterned to develop the thin structures, e.g., the working electrodes, access pads, the tracks. A similar lithography procedure was performed to produce a cover layer for the thin structures. The unprotected gold seed layer was etched by gold etchant for 60 sec followed by Cr etching using chromium etchant for 60 sec. The substrate was rinsed with DI water and SU-8 was stripped.

Ordyl SY390 (Elga Europe s.r.l., Italy) DFR was used to define the fluidic channel side walls, following a reported protocol.^[2] The surface of the wafer was first activated in an O₂-plasma cleaner at 0.3 mbar, 40 °C and 20 W at 40 kHz to improve the adhesion of the first laminated layer. Four fresh layers of Ordyl were laminated onto the glass substrate using a standard lamination device (Mylam 12, GMP) at 85 °C and roll speed 1 cm s⁻¹, resulting in a total nominal resist thickness of 90 µm. A 1 min post-lamination bake was performed at 85 °C on a hotplate. The Ordyl stack was then exposed to 365 nm UV light for a total dose of 180 mJ cm⁻² at 365 nm. A post exposure bake was performed for 1 minute at 85 °C.

The Ordyl patterns were developed for 5 min in Ordyl developer under ultrasonic agitation. The developed structures were rinsed in IPA and DI water and dried under nitrogen flow. Bonding to a 210 µm thick top glass wafer was achieved using a hot embossing device (EVG 510HE) with an applied pressure of ~6 bar, at a temperature of 95 °C for 30 minutes. Inlet and outlet holes were drilled into the top glass wafers before bonding, by machining the thin glass with a nanosecond UV laser (PIRANHA® ACSYS) pulsed at 30 kHz. The entire fabrication process is illustrated in Figure S1.

The sidewall electrode microfluidic device was fabricated as described previously.^[29]

1.3 Chitosan functionalization

Chitosan modification was always performed in solution (i.e. pH 5.0). After each modification, the resulting polymer was purified by precipitation with NaOH and dialysis against PBS (3 fold by volume) for at least one day at 4 °C before either the next coupling reaction, chemical analysis, or electrodeposition.

Polyethylene glycol (PEG) coupling. Chitosan was coupled with PEG featuring diacid functionality as described previously.^[25] PEG was activated with EDC and NHS in 20 mL of deionized water for 30 minutes at 24 °C. The PEG:EDC:NHS ratios were 1:0.5:0.5. This solution was then added to a 1 % w/v chitosan solution containing the appropriate quantity of amine reaction centers (PEG:CS-amine 1:1). The reaction was allowed to proceed for 3 hours at 24 °C before purification.

Quantum dot coupling. To aid in hydrogel layer visualization, quantum dot (QD) fluorescence was used. CS-QD samples were prepared as described previously.^[25]

Enzyme coupling. After dialysis of CSPEG, 0.6 g CSPEG hydrogel were weighed. This amount was chosen because of its good solubility in 3 mL of D₂O. The CSPEG was activated with EDC and NHS in 1 mL deionized water for 30 minutes at 24 °C. The ratios CSPEG:EDC:NHS were set to 1:0.5:0.5. Then each enzyme (Urs: 2 mg; GOx: 4 mg;

Figure S1: Illustration of the fabrication process for the planar electrode microfluidic platform with two different thicknesses of the working electrode, 3 nm and 34 nm Au. Each gold working electrode covers a nominal area of 1.76 mm^2 .

catalase: 1 mg) was mixed with this solution in a separate vial. The reaction was carried out at 24°C for 3 hours before a purification step. Purification was performed by overnight dialysis of the solution with PBS.

For the kinetic study involving Urs, 0.6 g of enzyme-bound CSPEG hydrogel (CSPEG-Urs) were weighed. The final sample was prepared by transferring 1 mL of the 1000 mM urea stock solution into an Eppendorf vial containing the CSPEG-Urs hydrogel. 500 μL of the obtained solution were transferred into an NMR tube. A timer was started at this point to track the inter-time t_0 between the start of the reaction and the acquisition of the first NMR spectrum. The same procedure was followed for kinetic studies of the GOx enzyme, mixing 0.6 g (CSPEG-GOx, CSPEG-cat) of hydrogel with 1 mL 100 mM of D-Glucose stock solution.

1.4 Enzyme coupling efficiency by UV absorbance

To determine the coupling efficiency of each enzyme to CSPEG, we performed UV absorbance measurements on samples containing the modified polymer and the enzyme. First, six different concentrations of free enzymes (Urs and GOx) were measured in three repeated experiments, as shown in Figure S2a and d. A series of six CSPEG-enzyme samples were prepared at various initial enzyme concentrations. After purification, UV absorbance was measured of the CSPEG-enzyme solution, as plotted in Figure S2b and e. To estimate the coupling yield, the absorbance measure-

ment of the CSPEG-enzyme was compared to the calibration plot obtained using free enzymes in solution. In the work reported in the main text, Urs was coupled to CS using an initial enzyme concentration of 2 mg mL^{-1} ($3.7 \mu\text{M}$), while GOx coupling was performed at 4 mg mL^{-1} ($25 \mu\text{M}$). The quantity bound under these initial conditions is highlighted by the arrows in Figure S2c and f. The resulting estimated coupling efficiency for Urs and GOx were 41% and 35%, respectively.

Figure S2: UV absorption spectra of (a) Urs, (d) GOx free in solution; (b) CS-Urs, (e) CS-GOx after coupling using the initial enzyme concentrations noted in the legend; (c) Urs and (f) GOx absorbance plots of the six calibration solutions (mean $\pm$ standard deviation of three replicates). Arrows indicate the absorbance of the purified CS-enzyme measured at the initial enzyme concentration used throughout this work (2 mg mL^{-1} Urs, 4 mg mL^{-1} GOx). Absorbance was measured at 280 nm (Urs, solution), 380 nm (GOx, solution), 300 nm (CS-Urs), and 400 nm (CS-GOx).

1.5 Chitosan electrodeposition

The chitosan solution (1% w/v, pH 5.5) was prepared by dissolving chitosan in a diluted HCl (0.2 M) solution as previously described.^[4,25] The solution was filtered through a 5 μ m syringe filter before use.

The channel was first cleaned with ethanol and washed thoroughly with DI water, followed by drying under nitrogen stream. Chitosan electrodeposition was performed in open and a closed configuration. The open configuration allowed for profilometric measurement of hydrogel height, while all NMR experiments were done using the closed configuration. In the open configuration the electrode array was directly exposed to the chitosan solution whereas in the closed system the chitosan solution was injected into the fluidic channel enclosing the electrode array. The electrodes were connected to a precision current source using the on-chip gold metal pads.

A PID-stabilized current density of $\sim 3 \text{ A/m}^2$ was run through the chosen electrode pair for a certain time to obtain a deposited layer of desired height. After electrodeposition, the chitosan solution was flushed and the deposited assembly was rinsed with DI water.

1.6 Hydrogel assembly

To ensure consistency in experimental conditions, the preparation of solution containing the enzymes and either coupling or not coupling to the CSPEG polymer were carried out according to the same protocol as we carried out using a standard 5 mm NMR saddle coil, except for the final target sample volume. In this case the sample volume is defined by the fluidic channel of the sample insert, that is 3.2 μ L for planar electrode and wide channel sidewall electrode configurations, and 2.5 μ L for the narrow channel sidewall electrode.

Relevant sample solutions:

- Solution A: 1000 mM urea
- Solution B: 100 mM glucose
- *Solution C: 35 μ M CS-Urs (15 U in 10 μ L)
- *Solution D: 20 μ M CS-Cat (1 U in 10 μ L)
- *Solution E: 32 μ M CS-GOx (10 U in 10 μ L)

(*Note: This is the expected number of units assuming 100% coupling yield)

A stock solution of 200 mM NaAc buffer at pH 7, and 50 mM TSP as NMR internal reference was used to prepare Solutions A and B to stabilise the pH and provide an NMR chemical shift reference at 0 ppm. Solutions C, D and E were filtered through a 5 μ L polycarbonate filter, and filled into three separate syringes.

Electrodeposition of the CS-Urs layer: Solution C was injected into the microfluidic channel, then electrodeposition was carried out for 15 minutes by running a PID-stabilized DC-current (current density of 3 A/m^2) through the chosen electrode pair to obtain a deposited layer of desired height (15–20 μ m). After electrodeposition, the enzyme solution was flushed and the channel was rinsed with D_2O . Then Solution A was injected and the inlet and outlet were immediately sealed. The sample insert was then mounted on the probe and the probe was transferred to the NMR magnet for real-time monitoring. The same procedure was followed by for deposition of solutions D and E.

Electrodeposition of multilayered chitosan assembly: the assembly was prepared using the following protocol: 1) injection of solution C, 2) electrodeposition + D_2O rinsing, 3) injection of solution D, 3) electrodeposition + D_2O rinsing, 4) injection of solution E, 5) electrodeposition + D_2O rinsing. Each deposition step was carried out for 15 minutes with the same current density (3 A/m^2) to obtain a deposited layer of the desired height (15–20 μ m).

Once the assembly was complete, a solution containing all substrates (1000 mM urea, 100 mM glucose, 50 mM TSP) was injected into the microfluidic chip, the inlet and outlet were immediately sealed, and the sample insert was then mounted on the probe and the probe was transferred to the NMR magnet for real-time monitoring.

1.7 Enzymatic activity using standard NMR

Enzyme in solution: two different samples were used in a standard 5 mm NMR tubes. The first sample was used to adjust the NMR parameters. These samples contained only the substrate, prepared in Eppendorf vials starting from a 1000 mM urea and a 200 mM glucose stock solution (in 200 mM NaAc buffer, pH 7.0). For urea samples the stock was used to prepare 1000 mM, 500 mM, 250 mM and 100 mM samples with a final volume of 1 mL each. For glucose samples, the stock solution was diluted to 100 mM, 50 mM, 30 mM and 10 mM with a final volume of 1 mL each. A 500 μ L volume was taken from each sample and transferred to a 5mm NMR tube.

The second sample was dedicated to the kinetic experiments. It contained 1000 mM urea and 2.0 mg/mL (3.7 μ M) urease. The sample was prepared by transferring 1 mL of the 1000 mM urea stock solution into an Eppendorf vial loaded with 2 mg dry stock enzyme. After thorough mixing, 500 μ L of the solution were transferred to an NMR tube for acquisition. A timer was started immediately after mixing the stock solution with the dry enzyme to track the time between the start of the enzymatic reaction and the acquisition of the first usable NMR spectrum. We labelled this intertime as T_0 . The same procedure was used for the kinetic study of the GOx enzyme. In this case a stock solution with 100 mM glucose was prepared and 1 mL of the stock were mixed with 3 mg/mL (19 μ M) dry GOx and 1 mg/mL catalase in an Eppendorf vial.

Enzymes coupled to chitosan: after dialysis of CS-modified polymer, 0.6 g of the hydrogel were weighed. The final sample was prepared by transferring 1 mL of the 1000 mM urea stock solution (in 200 mM NaAc buffer, pH 5.5) into an Eppendorf vial containing the CS-Urs hydrogel. 500 μ L of the obtained solution were transferred into an NMR tube. A timer was started immediately after mixing to track the time T_0 between start of the reaction and acquisition of the first NMR spectrum. The same procedure was followed for kinetic studies of the GOx enzyme, mixing 0.6 g (CS-GOx, CS-Cat) hydrogel with 1 mL 100 mM glucose stock solution.

1.8 Real-time NMR measurements

A custom designed NMR probe was employed featuring the integration of all sub-assemblies and providing the external connections for fluidics and NMR signal lines. The diameter was constrained by the magnet bore, a 500 MHz wide bore system (Bruker BioSpin, Rheinstetten) with a bore diameter of 72.9 mm. To provide flexibility for the fluidic interconnections we integrated a feed-through system that enabled the integration of tubings of variable diameters and established the connections of the micro-detector with the external control and supply components.

The probe was equipped with the required mechanical, electrical and fluidic connections to accommodate a commercial 3-axis micro gradient system. For final experimental installation, the gradient was slipped over the probe head and a tight mechanical locking mechanism ensured the rigid mounting of the probe head to the probe. The interface for the exchangeable NMR probe head provided the contacts for the ^1H RF channel. The probe was equipped with two additional high quality air trimmer capacitors (TG 092 Temex-Ceramics) that established a low loss interface between the 50 Ω transmission line and the RF resonator. A non-conducting glass fibre rod was mounted on each trimmer capacitor which enabled to conduct a final tuning and matching procedure once the final assembly was mounted into the MR scanner.

The experiments were performed with two different NMR coils. As a reference, control experiments were performed using a standard 5 mm saddle coil insert and Micro 5 micro-imaging probe (Bruker). Microfluidic experiments

were performed using a 1.2 mm Helmholtz microcoil.^[5] Electrodeposition of the CS-enzymes was performed outside of the magnet.

As control experiments, a one-dimensional (1D) NMR experiments were performed on the substrate (urae, glucose) and product (gluconic acid) solutions at 20 °C to identify the relevant signals. A standard pulse calibration sequence (nutration sequence) was performed to determine the 90° pulse. All chemical shifts are expressed in units of parts per million (ppm) with respect to TSP (0 ppm). Each 1D NMR spectrum was collected with a spectral width of 18.93 ppm (9469.70 hertz) over 34,000 data points for a total FID measurement time of 1.80 s. The relaxation delay was set to 1 s, to ensure that as many intermediate spectra as possible could be recorded before the complete enzymatic conversion of urea into the products. Averaging was performed with 8 scans for saddle coil and 200 scans for the Helmholtz microcoil. For the real-time measurements, the experiment was set up to collect FIDs continuously in order to follow the reaction. A delay of 10 minutes was added between NMR acquisitions, to allow for a reasonable time resolution and to limit the amount of data points (spectra) to be post-processed. This delay was added for convenience and could certainly be neglected in case higher time resolution is required.

Experiment	V_{max} mM/min	K_M mM	Container Volume μ l	Coupling Efficiency -	Theoretical Units U	Active Units U	Active Fraction %
Urs	Urs + urea	24 $\pm$ 7	712 $\pm$ 95	500 $\pm$ 6	-	100 $\pm$ 1.2	23.9 $\pm$ 5.8
	CS-Urs + urea	25 $\pm$ 5	601 $\pm$ 123	500 $\pm$ 6	0.41 $\pm$ 0.01	41 $\pm$ 1.1	62.0 $\pm$ 11.8
	Urs / GOx* + glucose, urea	17 $\pm$ 3	201 $\pm$ 21	500 $\pm$ 6	-	100 $\pm$ 1.2	17.0 $\pm$ 3.0
	CS-Urs / CS-GOx* + glucose, urea	19 $\pm$ 4	102 $\pm$ 10	500 $\pm$ 6	0.41 $\pm$ 0.01	41 $\pm$ 1.1	46.3 $\pm$ 5.1
GOx	GOx + glucose	2.5 $\pm$ 0.2	228 $\pm$ 50	500 $\pm$ 6	-	200 $\pm$ 2.4	0.6 $\pm$ 0.1
	CS-GOx + glucose	2.5 $\pm$ 0.2	50 $\pm$ 12	500 $\pm$ 6	0.35 $\pm$ 0.01	70 $\pm$ 2.2	1.25 $\pm$ 0.05
	Urs / GOx* + glucose, urea	2.4 $\pm$ 0.1	28 $\pm$ 6	500 $\pm$ 6	-	200 $\pm$ 2.4	0.60 $\pm$ 0.03
	CS-Urs / CS-GOx* + glucose, urea	2.6 $\pm$ 0.3	30 $\pm$ 9	500 $\pm$ 6	0.35 $\pm$ 0.01	70 $\pm$ 2.2	1.9 $\pm$ 0.2

Table 3: Summary of the kinetic activity of **Urease** and **Glucose Oxidase** using using the commercial 5 mm saddle coil. Kinetic parameters were extracted by fitting the consumption of urea and the production of gluconic acid, respectively. *Note: reactions of GOx were always done together with Cat in a 4:1 ratio.

Experiment		Gluconic acid		Glucose	
		V_{max} mM/min	K_M mM	V_{max} mM/min	K_M mM
Solution	GOx+D-Glucose	0.98 $\pm$ 0.04	280 $\pm$ 70	1.8 $\pm$ 0.8	110 $\pm$ 40
	CS-GOx+D-Glucose	0.87 $\pm$ 0.04	80 $\pm$ 20	1.5 $\pm$ 0.2	35 $\pm$ 10
	Urs/GOx+D-Glucose, Urea	1.5 $\pm$ 0.5	38 $\pm$ 6	0.9 $\pm$ 0.1	37 $\pm$ 6
	CS-Urs / CS-GOx + D-Glucose, Urea	0.9 $\pm$ 0.3	25 $\pm$ 5	0.9 $\pm$ 0.1	22 $\pm$ 3
Hydrogel	CS-GOx+D-Glucose	0.85 $\pm$ 0.05	60 $\pm$ 10	1.03 $\pm$ 0.09	16 $\pm$ 4
	CS-Urs / CS-GOx + D-Glucose, Urea	0.9 $\pm$ 0.1	22 $\pm$ 4	1.0 $\pm$ 0.2	7 $\pm$ 2
Planar electrode	CS-Urs / CS-GOx	0.9 $\pm$ 0.1	22 $\pm$ 4	1.0 $\pm$ 0.2	7 $\pm$ 2
Sidewall electrode	CS-Urs / CS-GOx	0.8 $\pm$ 0.1	23 $\pm$ 8	1.7 $\pm$ 0.8	25 $\pm$ 5

Table 4: Comparison of the GOx kinetic parameters extracted using the Michaelis-Menten progress curve fitting based on either gluconic acid production or glucose consumption.

2 Supporting Information 2: Results

2.1 Sequential electrodeposition of labelled CS using planar and sidewall electrode configurations

Figure S3: (a) Unfiltered (top) and filtered (bottom) fluorescence microscopy images and (b) mean profile height and fluorescence intensity charts versus deposition time as measured by profilometry and fluorescence microscopy for a three-step sequential electrodeposition of chitosan over 40 minutes total deposition time. A total of four hydrogel layers of QD-labelled chitosan ($\lambda_{\text{emission}} = 532 \text{ nm}; 560 \text{ nm}; 700 \text{ nm}$) were assembled

Planar electrodes: three layers of CS-QD were assembled as shown in Figure S3a with a 20 minute deposition time for each layer at a current density of 3 A/m^2 .

The fluorescence intensity from the gel layers was visualized layer by layer using fluorescence microscopy and the mean intensity was extracted using ImageJ. The intensity was then plotted versus time of deposition (Figure S3b). Meanwhile, to determine the thickness of the individual layers, the upper glass was removed from the platform to measure the thickness of the hydrogel layers using a profilometer. The results of the thickness measurement are shown in Figure S3b.

According to Figure S3b, for every 20 minutes of deposition, the thickness increased by 20-25 microns, together with a proportional increase in the mean fluorescence intensity, whereby the average intensity increased by a factor of 2. Individual layers can be visualized using an emission band pass filter, so long as individual layers are labeled with QD with different emission wavelengths.^[25]

Sidewall electrodes: the mean fluorescence intensity was extracted using ImageJ. The result shown in Figure S4c showed that the mean intensity was in the same range ($\sim 45 - 55 \pm 5 \text{ a.u.}$). In the sidewall configuration, profilometer measurement of hydrogel thickness was not possible, and therefore standard light microscopy was used. For each layer, a measurement ruler was placed to estimate the width of the layer, the results are presented in Figure S4c.

Figure S4: Image from (a) optical microscopy; (b) unfiltered fluorescence microscopy and (c) Mean profile height and fluorescence intensity charts versus time deposition as measured by optical microscopy and fluorescence microscopy for a four-step sequential electrodeposition of chitosan over 40 minutes total deposition time. A total of four hydrogel layers of QD-labelled chitosan ($\lambda_{\text{emission}} = 532 \text{ nm}$; and 700 nm) were assembled

Figure S5: pH for (a) Urs and CS-Urs; (b) GOx and CS-GOx and (c) multiplex with and without polymer. Reactions were performed in NaAc buffer, pH 5.5.

Figure S6: Schematic of the hydrogel layers for individual enzyme deposition and multiplex deposition conditions using (a) planar electrode geometry and (b) sidewall electrode geometry.

2.2 pH measurement during catalysis

2.3 Calculation of hydrogel volume

Figure S6 shows the estimated volume of each hydrogel layer for the two electrode configurations; (a) planar electrode and (b) sidewall electrode. For the planar electrode (Figure S6a), thickness was determined for both the Multilayered Assembly and Individual Layer configurations. The thickness of the hydrogel (t_{gel}) was determined using the profile measurement method, performed by removing the upper glass of the channel. The values of t_{gel} are the thicknesses of the individual hydrogel layers (i.e. not the cumulative thickness).

For the sidewall electrode (Figure S6b), the thickness of the hydrogel layers was estimated by light microscopy. The thickness was measured after preparing the multilayered CS-Urs / CS-Cat / CS-GOx hydrogel assembly, using the same current density and deposition time as in the planar electrode case. The values of t_{gel} are the thicknesses of the individual hydrogel layers (i.e. not the cumulative thickness).

2.4 Calculation of enzyme active fraction

Theoretical unit Urs in solution

$$U_{Urs, \text{theoretical}} = \frac{200 \text{ unit}}{1 \text{ mL}} \times \text{volume of microfluidic channel}$$

Active unit Urs based on V_{max}

One mole of urea produces two moles of ammonia. The extracted V_{max} of the reaction gives M_{urea}/min , which corresponds to $M_{NH_3}/\text{min} \times 0.5$. Then the active unit can be estimated by multiplication with the microfluidic channel volume of the reaction (3.5 μL for the planar electrode and 2.5 μL for the narrow channel sidewall electrode).

$$U_{Urs, \text{active}} = (V_{max} \times 2) \frac{M_{NH_3}}{\text{min}} \times \text{Volume of reaction}$$

Active unit GOx based on V_{max}

One mole of glucose produces one mole of gluconic acid. V_{max} of the reaction yields $M_{\text{gluconic acid}}/\text{min}$, which corresponds to a of one mole of $M_{\text{gluconic acid}}/\text{min}$. Therefore it is directly calculable to estimate the active unit by multiplying the reaction volume.

Theoretical unit CS-enzymes in hydrogel

$$U_{\text{theoretical}} = \frac{\text{initial theoretical units in channel} \times \text{coupling efficiency}}{\text{volume of hydrogel}}$$

Active units of CS-enzymes in hydrogel gel based on V_{max}

$$U_{\text{CS-Urs, active}} = (V_{\text{max}} \times 2) \frac{M_{\text{NH}_3}}{\text{min}} \times \text{Volume of hydrogel}$$

$$U_{\text{CS-GOx, active}} = (V_{\text{max}}) \frac{M_{\text{gluconic acid}}}{\text{min}} \times \text{Volume of hydrogel}$$

Fraction of active units

$$\frac{\text{active unit}}{\text{initial unit}} \times 100\%$$

2.5 Reproducibility

Figure S7: Experiment reproducibility. Extracted concentration results from ^1H NMR spectra of the multi-layered hydrogel assembly CS-Urs / CS-Cat / CS-GOx exposed to a solution of 100 mM glucose, 1000 mM urea and 50 mM TSP. The experiment was performed 3 times using the planar (a) and sidewall (b) electrode configurations. Plotted are the urea (i), glucose (ii), and gluconic acid (iii) concentrations as a function of time.

2.6 Solution experiments using Helmholtz microcoil

Figure S8: **Urease** and **CS-Urs** solution experiments. ^1H NMR spectra of (a) Urs; (b) CS-Urs, each with 1000 mM of urea in solution with 50 mM of TSP as a reference and (c) extracted urea concentrations from the NMR results in (a) and (b). Closed symbols: Urs in solution, open symbols: CS-Urs in solution. All solutions were prepared in 200 mM NaAc, pH 5.5.

Figure S9: **Glucose oxidase** and **CS-GOx** solution experiments. ^1H NMR spectra of (a) GOx; (b) CS-GOx, each with 100 mM glucose in solution with 50 mM of TSP as a reference and (c) extracted concentrations from the NMR results in (a) and (b). Closed symbols: GOx in solution, open symbols: CS-GOx in solution. All solutions were prepared in 200 mM NaAc, pH 5.5.

Figure S10: Urs, GOx, and Cat in solution. ^1H NMR spectra of the enzyme mixture in solution with 100 mM of glucose, 1000 mM urea and 50 mM of TSP as a reference and (b) extracted reagent and product concentrations from the NMR results in (a). Left axis: urea concentration; Right axis: glucose and gluconic acid concentration. All solutions were prepared in 200 mM NaAc, pH 5.5.

Figure S11: CS-Urs, CS-GOx, and CS-Cat in solution. ^1H NMR spectra of the CS-enzyme mixture with 100 mM of glucose, 1000 mM urea and 50 mM of TSP as a reference and (b) extracted reagent and product concentrations from the NMR results in (a). Left axis: urea concentration; Right axis: glucose and gluconic acid concentration. All solutions were prepared in 200 mM NaAc, pH 5.5.

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