Electronic Supplementary Material (ESI) for Lab on a Chip.

SUPPORTING INFORMATION

McCLEC, a robust and stable enzymatic based microreactor-platform

Mayte Conejero-Muriel,[a] Isaac Rodríguez-Ruiz,[b] Sergio Martínez-Rodríguez,[c] Andreu Llobera[b] and José A. Gavira*[a]

Microdevice fabrication.

PDMS McCLECs were fabricated in one single step by casting of PDMS in a 2 level SU-8 master.[1] The technology is very simple and it has been deeply studied and reported, but it will be briefly described for completeness. After cleaning a low-cost 500 mm thick soda-lime glass wafer, it is dehydrated at 200 ºC for 1 h prior to the spinning of an SU-8 layer with a thickness of 5 µm, which act as a seed layer for the subsequent SU-8 layers. Afterwards, the substrates are baked at 95 ºC for 10 minutes and exposed to UV light without using a mask and a post-exposure bake (PEB) at 95 ºC is followed, with a slow cooling down to room temperature, in order to avoid any crack in the layer due to temperature stress. Then, with a single spin-on process using SU-8 2050, a thickness of 250 µm is obtained. A subsequent 2.5 h bake to remove the solvent from the SU-8 layer is performed prior to the UV exposition with the mask that describes the channels of the main microfluidic structure of the device (inlet and outlet ports, zigzag mixer, serpentine channel and filters). A PEB and the spinning of a second 200 µm SU-8 layer are followed. The thickness of this layer defines the depth of the microwells, which form the solution trapping system, which structures will be UV-patterned after another prebake and UV exposition. Finally, a last PEB is followed by the development of the structures in PGMEA, finishing the definition of the master. To end with the master fabrication process and to increase its long-term life for future microfluidic device fabrication, a 2 h hard bake at 120 ºC relieves the mechanical stress of the master and heals possible microcracks in the structure.

The PDMS pre-polymer is obtained by mixing the curing agent with the elastomer base in a 1:10 ratio (*v:v*). The resulting mixture is subsequently degassed to remove air bubbles, and a thin layer (between 0.5 and 1 mm) is poured over the master and cured for 60 min at 80 ºC. Afterwards, the cured PDMS is peeled off from the master and the fluidic ports are opened. Then, both the PDMS and a second soda- lime glass substrate are exposed to oxygen plasma [2] in a barrel etcher (Surface Technology Systems, Newport, UK). Immediately afterwards, both surfaces are brought in contact, causing its irreversible sealing. This covalent bonding between the PDMS and the soda-lime glass ends the fabrication process for the PDMS devices. A schematic representation of the process is depicted in Fig. S1.

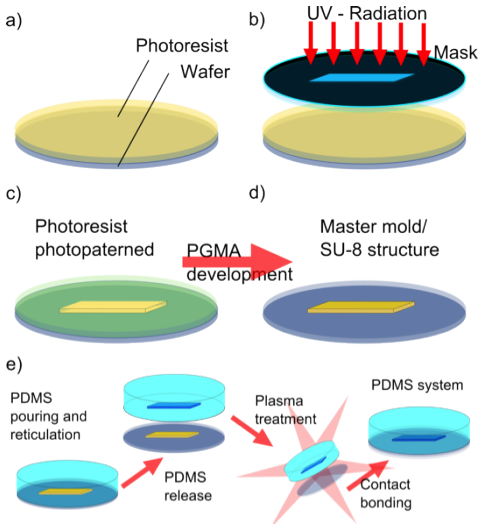


Fig. S1. (a) - (e) Schematic representation of the fabrication of a SU-8 mold and the casting and fabrication of a PDMS based microfluidic system.

Adaptation of FASE crystallization conditions to McCLFCs.

Initial counter diffusion FASE crystallization conditions were adapted to batch method in two steps. Firstly micro-batch under oil was used to reduce protein and precipitant concentration to desirable nucleation density and crystals size values. Table S1 shows the initial grid screen performed for FASE crystallization by varying the concentration of the protein from 15 to 3.25 mg.ml-1 and that of precipitant agent from 50% to 12.5% using paraffin oil and stored the experiments at 293 K. We observed that crystals obtained using a final protein concentration between 7.5-3.25 mg.ml-1 and 50% of MixPEG as precipitant agent could adjust to the desirable crystallization in McCLECs; thus, as second step, we reproduced and adjusted the same conditions using batch experiments in Eppendorf-tubes (Fig. S2A) and crystals with a size ranged in the McCLECs wells dimensions were obtained. After that, the experiments were set up in volumes ranging from 10 to 500 µL to study the influence of the volume on the final nucleation density and crystal size.

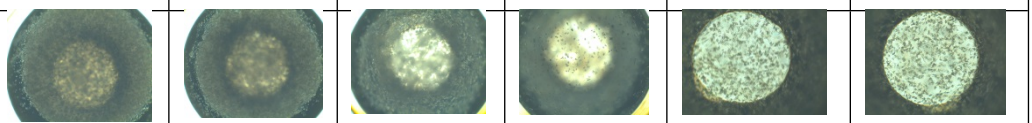
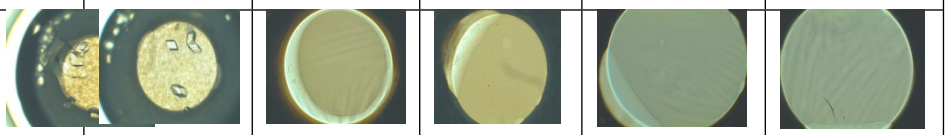
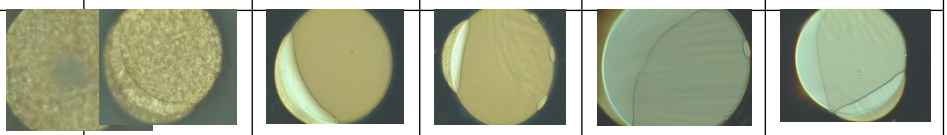
		FASE concentration (mg.ml^{-1})					
		15	15	7.5	7.5	3.	3.25
MixPEG pH 4.0	50%						
	25%						
	12.5%						

Table S1. Initial FASE crystallization grid obtained by varying protein precipitant agent concentrations by microchatch under oil method at 293K.

After the optimization of the FASE crystallization condition by batch method, crystals obtained in Eppendorf-tubes were cross-linked using 1% glutaraldehyde during 1 hour, producing CLFCs as shown in Fig. S2B. Resulted CLFCs were extensively washed with water, lyophilized and used for enzymatic assays.

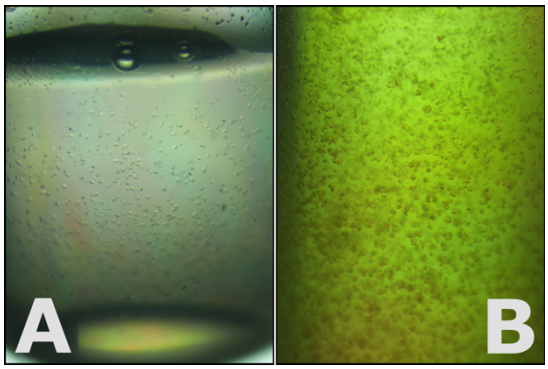


Fig. S2. A) FASE crystals obtained by batch method in Eppendorf-tubes with a final protein concentration of 5 mg.ml^{-1} and 50% precipitant agent (MixPEG pH4.0). B) CLFCs obtained by cross-linking of FASE crystals obtained by batch method in Eppendorf-tubes by using 1% glutaraldehyde for 1 hour at room temperature.

Determination of CLECs protocol formation using Lysozyme crystals.

A cross-linking protocol for CLECs was established using lysozyme crystals in different steps. Firstly, the minimum amount of lysozyme crystals necessary for a perfect visualization by SDS-PAGE was determined. For this purpose, lysozyme crystals were grown using a final protein concentration of 30 mg·ml^{-1} and 2.5% of NaCl as precipitant agent using the sitting drop crystallization technique in micro-bridges at 293K (Fig. S3A). Different isolated lysozyme crystals were then fished out, dissolved, boiled at 373 K in loading buffer and visualized in SDS-PAGE gels. 8 lysozyme crystals were estimated as the minimum amount of protein to be dissolved for SDS-PAGE analysis (Fig. S3B). Secondly, different cross-linking experiments were performed using 1, 3 and 5% of glutaraldehyde as bifunctional cross-linker and different incubation times with lysozyme crystals. In all cases, lysozyme crystals were fished out from their crystallization micro-bridges, washed with 5% NaCl (hypertonic solution to avoid the damage of the crystals) and finally placed in a new 5% NaCl drop on a Petri dish (Fig. S3A). Glutaraldehyde was added to different drops (1, 3 and 5%) and 8 lysozyme crystals were fished out at different times during 2 hours. The fished lysozyme crystals were placed in an eppendorf-tube with 20 µl of water, centrifuged and boiled for their visualization by SDS-PAGE. As result, 1% glutaraldehyde and 45 minutes were sufficient to avoid crystal dissolution and therefore this condition was selected for CLLCs generation (Fig. S3B).

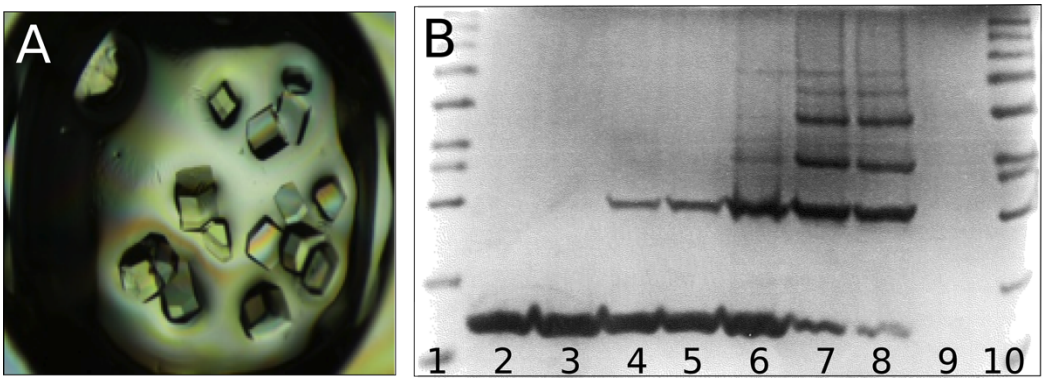


Fig. S3. SDS-PAGE analysis of cross-linked lysozyme crystals (CLLCs) with 1% glutaraldehyde fished out at different incubation times. A) Lysozyme crystals during the cross-linking process in a 5% NaCl drop on a Petri dish. B) Cross-linking of lysozyme crystals with 1% glutaraldehyde incubated for: Lane 2, 30 seconds; Lane 3, 1 minute; Lane 4, 2 minutes; Lane 5, 5 minutes; Lane 6, 10 minutes; Lane 7, 20 minutes; Lane 8, 30 minutes; Lane 9, 45 minutes; Lanes 1 and 10, molecular-mass marker.

Comparative enzymatic study of soluble proteins vs CLECs.

The enzymatic assay for lysozyme was adapted from Osawa T. experiments.[3, 4] Colorimetric determination of p-nitrophenol liberated during the reaction was followed at 400 nm soluble lysozyme (0.100 mg) and the same amount of CLLCs obtained by vapour diffusion experiments and cross-linked with 1% glutaraldehyde during 45 minutes at RT were used. In both cases enzymatic assays were performed by shaking-incubation with nitrophenyl-β-D-N, N´, N´´-triacetylchitotriose (1 mg·ml^{-1}) dissolved in 1 mM sodium phosphate pH 9.0 for 2 hours at 293K. 19 independent enzymatic reactions were performed with soluble lysozyme (Fig. S4A blue bars). On the other hand, McCLLCs were reused 18 times (washed with water among the experiments) (Fig. S4A red bars).

Enzymatic production of ammonia by FASE was spectrophotometrically determined using a commercially available ammonia determination kit, following manufacturer instructions (Ammonia kit, R-Biopharma, 11112732035). This determination was performed using 0.300 mg of soluble FASE and the same amount of CLFCs obtained by batch experiments in Eppendorf-tubes at 293K and cross-linked with 1% glutaraldehyde during 45 minutes. In both cases enzymatic assays were performed by shaking-incubation with 6 mM formamide in 100 mM citrate/citric acid pH 6.0 for 2 hours at 293K. 20 different enzymatic reactions were performed with soluble FASE and CLFCs (Fig. S4B, blue and red bars, respectively), where a homogeneous behaviour is observed.

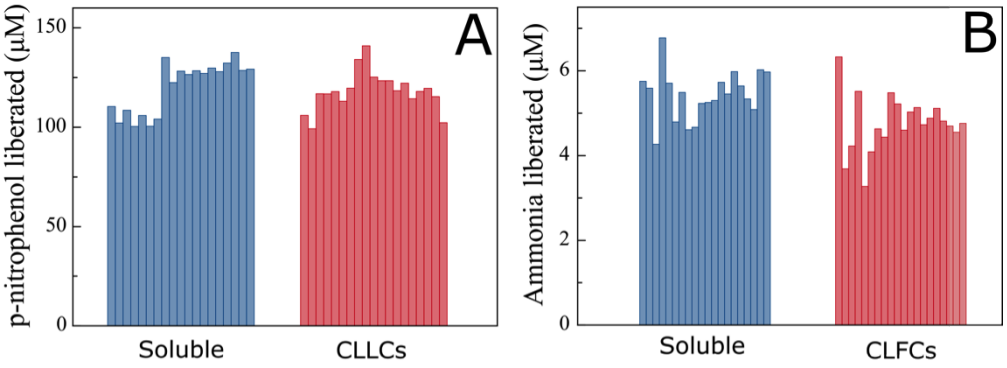


Fig. S4. Comparative activity assays with A) soluble lysozyme (blue bars) and CLLCs (red bars) and B) soluble FASE (blue bars) and CLFCs (red bars). For the soluble protein each bar represent an independent experiment while in the case of CLECs each bar correspond to a different cycle of the same set of CLECs.

McCLECs re-utilization.

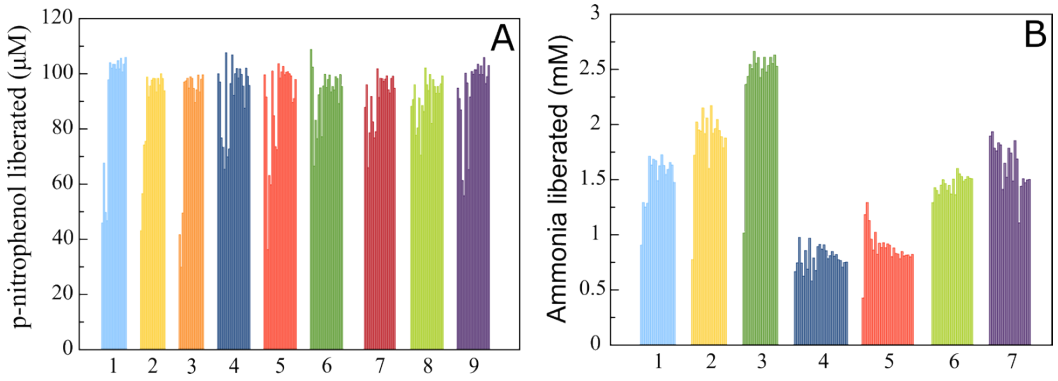


Fig. S5. A) Results of enzymatic assays with 9 different McCLLCs. McCLLCs 1-3 were initially used for activity proofs and then stored for one year after; McCLLCs 4-9 had been stored for 8 months. B) Results of enzymatic assays with CLFCs produced in 7 different microfluidic chips. McCLFCs 4-7 had been stored during 4 months dried at room temperature until use for enzymatic assays and McCLFCs 1-3 8 months after the initial proofs. In both cases, each colour corresponds to a different McCLEC and each bar represents an enzymatic re-use of the CLECs in each chip.

References

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