

# Lab on a Chip

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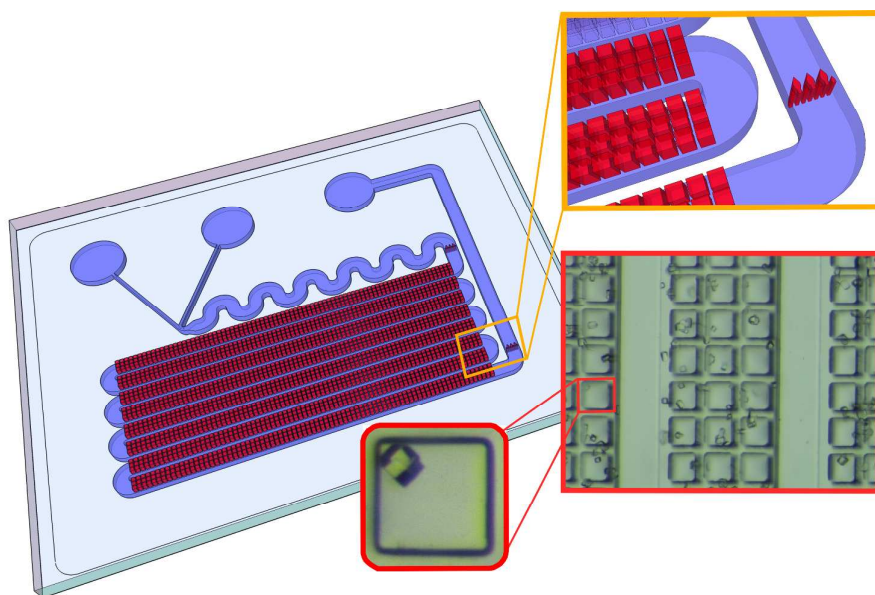
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## Graphical Abstract

### McCLEC, a robust and stable enzymatic based microreactor-platform

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We present a microfluidic chip, McCLEC, for the crystallization, cross-linking and enzymatic reactions. We demonstrate a high stability, robustness and reusability envisaging its promising use in biotechnological applications.





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## McCLEC, a robust and stable enzymatic based microreactor-platform

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A Microfluidic chip for Cross-Linked of Enzyme Crystals (McCLEC) is presented and demonstrated to be a stable, reusable and robust biocatalyst-based device with very promising biotechnological applications. The cost-effective microfluidic platform allows in situ crystallization, cross-linking and enzymatic reaction assays on a single device. A large number of enzymatic reuses of the McCLEC platform were achieved and a comparative analysis is shown illustrating the efficiency of the process and its storage stability for more than one year.

### Introduction

The use and development of enzymes as robust biocatalysts is one of the main challenges in biotechnology.<sup>1</sup> Enzymes are a specific kind of proteins involved in the catalysis of essential biochemical reactions in life processes. Biocatalysis allows the mild and selective formation of products using isolated enzymes. Compared to chemical methods, biocatalysis shows excellent chemo-, regio- and stereoselectivity while retaining a small environmental footprint. Enzymes are widely applied in many different industries such as food processing (for human and animals),<sup>2</sup> pharmaceuticals,<sup>3, 4</sup> material processing (textiles, paper, detergents, etc.), waste treatment and also to produce biofuels.<sup>5</sup> Similar level of development can be found in the application of enzymes in biosensor technologies.<sup>6-8</sup> However, there are also drawbacks in enzyme-catalyzed reactions. They can exhibit a slow reaction rate, be unstable in practical operational conditions (such as high temperatures or the use of organic solvents), their storage stability (or shelf life) can be poor and the downstream processing can be complex. At the industrial level these drawbacks are typically overcome by the production of immobilized enzymes to enhance stability, robustness and solubility.<sup>9</sup>

Immobilization is aiming as a very powerful tool to improve the characteristic properties of enzymes (stability, activity, specificity and selectivity), allowing continuous operation, the control of the enzymatic reaction and also enzyme recovering and reuse.<sup>10, 11</sup> Among all the immobilization techniques, the crosslinking of enzymes in their crystalline state, CLECs (Cross-linked Enzyme

Crystals) or as aggregates, CLEAs (Cross-linked Enzymes Aggregates), are being explored. Although CLEAs are the easier and economically beneficial way to produce these highly active materials, compared to CLECs, the particle size and distribution are difficult to control and they are less robust. CLECs, described for the first time in 1964 by Quijcho and Richards,<sup>12</sup> are prepared from the crystallization of the enzyme followed by cross-linking of the microcrystals produced by using bifunctional reagents.<sup>13</sup> In contrast to enzymes immobilized by classical techniques (binding to a support or encapsulation of the enzymes), carrier-free immobilized enzymes in the form of CLECs present numerous advantages versus carrier-bound or free enzyme: i) 10 to 1000 higher volumetric activities (U/g), as CLECs are basically pure protein, with a high concentration of enzyme per volume unit (the use of a carrier inevitably suffers from dilution of volumetric and specific activities as carriers can account for 90% to >99% of the mass or volume of the catalyst,<sup>14</sup> ii) higher stability against unnatural conditions (high temperature, organic solvents, etc.), as the enzyme crystal formation and the additional cross-linking process prevent denaturation, unfolding or degradation of the enzyme by proteases,<sup>14</sup> and iii) in particular cases even higher selectivity. Furthermore, CLECs can be easily lyophilized and indefinitely stored at room temperature and their insoluble nature facilitates the isolation, recycling and reuse of the enzyme.<sup>13</sup> Therefore, the use of CLECs for biosensing applications has a ground-breaking and yet unexplored potential when considering their actual storage limitations (biosensors cannot be stored for long periods due to quick denaturation of the enzymes) and shelf life, which usually last just a few days, or weeks in the best situation.

In spite of all the advantages mentioned above, crystallization is hitherto identified as the limiting step for the production of CLECs. However, taking advantage of microfluidics, it is possible to provide an unique environment for the production and immobilization of enzyme crystals. Microfluidics offer clear advantages over more conventional systems, such as a dramatic reduction of reagent

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consumption, shorter analysis time, separation efficiency and high resolution and sensitivity in detection.<sup>15-19</sup> Microfluidics have been also applied in protein crystallization<sup>20, 21</sup> and enzymatic assays, providing sample and reagent volumes smaller than those used in robotic systems,<sup>22-24</sup> and exploiting the short diffusion lengths inside the devices to have faster reaction velocities.<sup>25-27</sup> Additionally, this science field offers the attractive possibility of combination of chemical processes and biological assays in the same device.<sup>22, 28</sup> In this way, and thanks to the reduction of volume reagents (*i.e.* costs effective), in terms of costs, microfluidics, or lab-on-a-chip technology,<sup>29, 30</sup> is offered as a powerful and high throughput approach in a vast number of applications as molecular biology, protein analysis, immunoassays, diagnosis and drug development.<sup>31-40</sup> Indeed, the use of enzymatic catalytic reactions under microfluidic flow conditions reveals a promising technology with a series of strategic advantages, such as dramatic enhancements of surface/volume ratios and special/temporal reaction control, continuous processing at smaller scales, improved energetic efficiency and mass transport in multiphase reactions or faster process development, which are putting it into the spotlight of vast and broad applications, speeding up the discovery and development of new biotechnological applications.<sup>41</sup>

In this work we propose the use of Cross-Linked-Enzyme-Crystals (CLECs) for enzymatic catalytic reactions as a stable, reusable and robust catalyst, with very promising applications in the synthesis of high value products as well as in biosensing. For this purpose, and taking advantage of a common technology widely used in biosensing applications, a microfluidic platform, McCLEC (Microfluidic chip for Cross-Linked Enzyme Crystals), has been conceived for performing *in-situ* and spatially controlled enzyme crystallization from solution and to cross-link the crystals that can repetitively be used or feed with the appropriated substrate. As a proof of concept, the crystallization process, subsequent cross-linking and *in situ* enzymatic reaction of two proteins is here presented: lysozyme as model protein and a formamidase as a protein of interest for the pharmaceutical industry, for either the production of high value products or its use in sensing technologies.

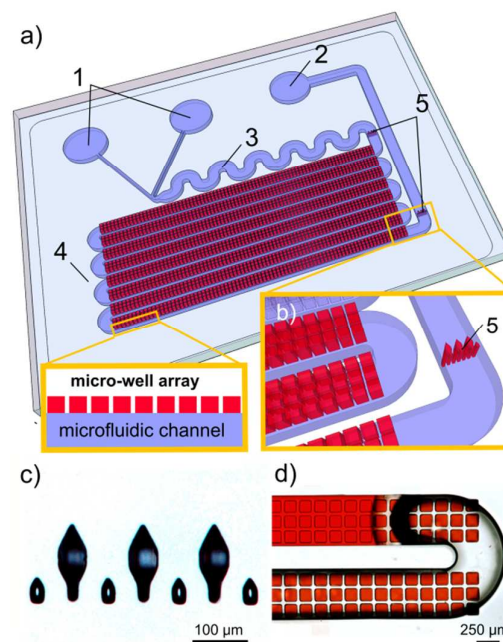
## Experimental

**Materials and reagents.** PDMS Sylgard 184 elastomer kit was supplied by Dow Corning (Midland, MI, USA). SU-8 negative tone photoresist and Propylene glycol methyl ether acetate developer (PGMEA) were supplied by MicroChem, Corp. (Newton, MA, USA).

Chicken egg-white lysozyme (lysozyme) was purchased as a lyophilized powder from Sigma (L6876). *Bacillus cereus* formamidase (FASE) was produced in a two-step-purification in the lab. Glutaraldehyde (G7651) and formamide (F4761) were purchased as solutions from Sigma and 4-Nitrophenyl-beta-D-N,N',N''-triacetylchitotriose from Santa Cruz Biotechnology (sc-220973A). Ammonia kit was purchased from R-Biopharma (11112732035).

**McCLECs design, concept and fabrication.** McCLECs were fabricated in one single step by casting of PDMS in a 2 level SU-8 master<sup>42</sup> (See supporting information for details and Fig. S1).

Their global design is shown in Fig 1 and it is described as follows: Two different inlet ports (number 1 in Fig. 1a) permit the mixing of up to 2 different solutions if required, which downstream merge in a single channel configured as a passive zigzag mixer (number 3 in Fig. 1a), which is implemented in the design for providing a fast homogenization of the mixtures<sup>43</sup>. Subsequently, a serpentine channel with a section of 250x500  $\mu\text{m}$  (number 4 in Fig. 1a), allows the stored of thousands of nanolitre droplets in each micro device by means of a solution trapping system. A detail of the system, consisting on an array of microwells with a volume of 2 nL each (100x100  $\mu\text{m}$  section, 200  $\mu\text{m}$  high) can be seen in Fig. 1b. The array is constructed over a main serpentine channel, but at a different height (inset in Fig. 1a). The serpentine channel containing these microwell structures provides a total reaction volume of  $\approx 17 \mu\text{L}$ , being the total McCLEC volume of  $\approx 20 \mu\text{L}$ . The operation of the trapping system will be lately described in the text. Additionally, a filter of PDMS was fabricated in the same single cast-molding step, which defines the microfluidic structure and wells (number 5 in Fig. 1 a and b). The filter is located before and after the serpentine channel so to prevent any possible non-fixed crystal or crystal aggregate larger than 30 microns to be dragged by the injected solutions, either during the cross-linking of the crystals or the subsequent enzymatic catalytic reaction. A detail of the PDMS filter is presented in Fig. 1c. Finally the serpentine channel ends up in an outlet port (number 2 in Fig. 1a), which is positioned at the same plane of the inputs, so to facilitate the microscope access to the reactor region.



**Fig. 1.** Images and schematics of McCLECs. a) General view: (1) inlet ports; (2) outlet port; (3) passive zigzag mixer; (4) serpentine channel for droplet storage; (5) PDMS filters; (Inset on the left): cross section depicting the layout of the solution trapping system (microwell array) over the microfluidic channel. b) Detail of the microwell array



and the PDMS filter; c) Image of the PDMS filter; d) Operation of the solution trapping system during the injection of a red dyed solution. The meniscus of the flowing solution is observed when emptying the microfluidic device, while the solution trapping system retains nanolitre-sized droplets in the microwell array.

**Protein preparation and production.** Lysozyme was dissolved and dialyzed in 50 mM sodium acetate pH 4.5. *B. cereus* formamidase (FASE) was expressed in *E. coli* BL21 (DE3) as a fusion protein with a C-terminal hexahistidine tag and purified from bacterial lysates to homogeneity, in two steps: *i*) affinity chromatography (15 ml bed volume, GE Healthcare) in 50 mM  $\text{Na}_2\text{HPO}_4$ , 300 mM NaCl buffer and *ii*) size exclusion chromatography (Superdex 200 column; GE Healthcare) in 20 mM Tris-HCl pH 8.0. The protein was then concentrated using a Centricon centrifugation system with a 10 kDa molecular mass cut-off membrane. Based on SDS-PAGE experiments, the purity of the recombinant protein was estimated to be greater than 95%.

**Crystallization experiments.** McCLECs were tested for crystallization with lysozyme as model protein and FASE as an enzyme with potential biotechnological applications.

An initial grid screening for lysozyme crystallization was performed by varying the concentration of the protein from 10 to 50  $\text{mg}\cdot\text{ml}^{-1}$  and that of precipitant agent NaCl from 1 to 10% in hanging drop experiments at 293 K. Although all the conditions of the grid produced crystals, the most suitable crystals, in size and number, were obtained at a concentration of protein of 20  $\text{mg}\cdot\text{ml}^{-1}$  and 5% of NaCl as precipitant agent. Thus, this crystallization condition was adapted for lysozyme crystallization in McCLECs by batch method, obtaining tetragonal lysozyme crystals at a supersaturation ( $C/C_{\text{Equilibrium}}$ ) of 4.2.

Initial crystallization conditions for FASE were obtained by screening in capillaries of 0.3 mm inner diameter using the counterdiffusion technique in pre-filled GCBs<sup>45</sup>. In this case, the precipitant agent was a mixture of PEGs (20% PEG 400, 15% PEG 4000, 10% PEG 8000) in the range of pH 4.0 to 9.0 (KIT PEG448-49, Triana S&T) and the protein was used at a concentration of 28  $\text{mg}\cdot\text{ml}^{-1}$ . Conditions were then adapted to batch method in two steps (Table S1, Fig. S2). Firstly microbatch under oil was used to reduce protein and precipitant concentration to a desirable nucleation density and crystals size values by varying protein concentration between 15 to 3.75  $\text{mg}\cdot\text{ml}^{-1}$  and diluting the precipitant cocktail from 50 to 12.5% with 100 mM sodium acetate pH 4.0 (Table S1); secondly similar conditions were used in Eppendorf-PCR tubes to produce FASE crystals that could be further cross-linked and recovered (Fig. S2). The experiments were set in volumes ranging 10 to 500  $\mu\text{L}$  to study the influence on the final nucleation density and crystal size.

Crystallization experiments in McCLECs were performed by batch technique in different steps. Initially, the McCLECs devices were incubated with a solution containing 1% of Triton

X-100 surfactant (108643 Merck Millipore) in order to modify the highly hydrophobic PDMS surface allowing the correct filling of the microwell array. The crystallizing batch solution (enzyme and precipitant agent) was injected to fill each McCLEC and subsequently a soft vacuum was applied through the outlet port emptying the main microfluidic channel. The difference in surface energy of both the modified PDMS walls and injected solutions makes possible to extract the excess of solution while keeping the microwell array filled (Fig. 2, step 1). McCLECs were stored in Petri dishes with water-reservoirs to avoid evaporation from the microwell array.

A total of 15-30  $\mu\text{L}$  of crystallizing solution was set up for each protein; 20  $\text{mg}\cdot\text{ml}^{-1}$  of protein and 5% NaCl as precipitant agent were used in the case of HEWL, whereas and 5  $\text{mg}\cdot\text{ml}^{-1}$  of protein and a polyethylenglycol mixture (10% PEG 400, 7.5% PEG 4000, 5% PEG 8000) in 100 mM sodium acetate pH 4.0 was used to trigger FASE crystallization. All the experiments were stored at 293 K and observed with an optical microscope. Pictures were acquired with a ProgRes® CapturePro 2.8 detector (JENOPTIK optical systems, GmbH).

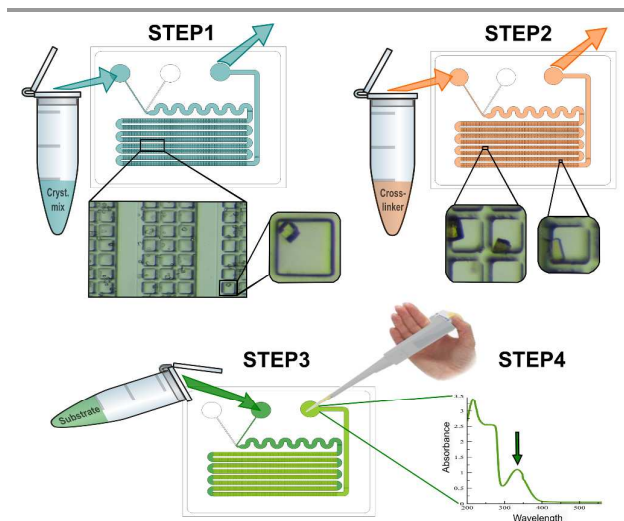


Fig. 2. Schematic representation of the three major steps carried out to prepare CLECs in McCLECs. Step 1 shows the crystallization process inside the device and examples of lysozyme crystals are depicted. Step 2 displays the cross-linking process showing the cross-linked crystals. Finally, steps 3 and 4 respectively show the operation of the chip as an enzymatic reactor and the final spectrophotometric detection for the product of the reaction.

**Cross Linked Enzyme Crystals production.** Crystals of both enzymes were cross-linked by incubating McCLECs for 1 hour at room temperature (RT) with the corresponding crystallizing solution (see above), supplemented with 1% (v/v) glutaraldehyde. Incubation time and cross-linker concentration were both previously optimized using SDS-PAGE electrophoretic analysis (see Supporting Information for details and Fig. S3). An example of cross-linked lysozyme crystals is shown in Fig. 2, step2. Subsequently, cross-linked crystals were carefully washed injecting at least three volumes of water into the McCLECs. Finally the McCLECs containing

cross-linked lysozyme and FASE Crystals (McCLCs and McCLFCs, respectively) were dried out and stored at RT for several months.

**Enzyme assays.** Spectrophotometric determinations were carried out in a Cary 300 UV-VIS spectrophotometer (Agilent technologies, Santa Clara, CA), with blank corrections for all measurements. The enzymatic assay for lysozyme was adapted from Osawa T experiments<sup>46,47</sup> to be assayed colorimetrically by using Nitrophenyl- $\beta$ -D-N, N', N''-triacylchitotriose as substrate and the p-nitrophenol liberated as product of the reaction was determined at a wavelength of 400 nm. Enzymatic production of ammonia by FASE was spectrophotometrically determined using a commercially available ammonia determination kit, following manufacturer instructions (Ammonia kit, R-Biopharma, 11112732035).

CLECs activities grown inside McCLECs were determined by reaction with 4-Nitrophenyl- $\beta$ -D-N, N', N''-triacylchitotriose ( $1 \text{ mg}\cdot\text{ml}^{-1}$ ) dissolved in 1 mM sodium phosphate pH 9.0 (McCLCs) and 6 mM formamide in 100 mM citrate/citric acid pH 6.0 (McCLFCs) (Fig. 2, step 3). Substrate-loaded McCLECs were incubated for 2 hours at 323 K in Petri dishes containing a water-reservoir to avoid solution evaporation from the devices. The reaction solutions were then recovered from the devices using a micropipette, and product concentrations were determined were spectrophotometrically as described above (Fig. 2, step 4).

In order to compare the activity of CLECs produced both inside and outside McCLECs, crystals for the two proteins were produced by the batch methods, cross-linked adding 1% glutaraldehyde directly in their mother liquor, washed, lyophilized, weighted onto a precision balance and added to the corresponding reaction solutions (see Supporting Information for details and Fig. S4). For the re-use evaluation experiments, CLECs were extensively washed with water after each run, dried out and stored at RT till the next use.

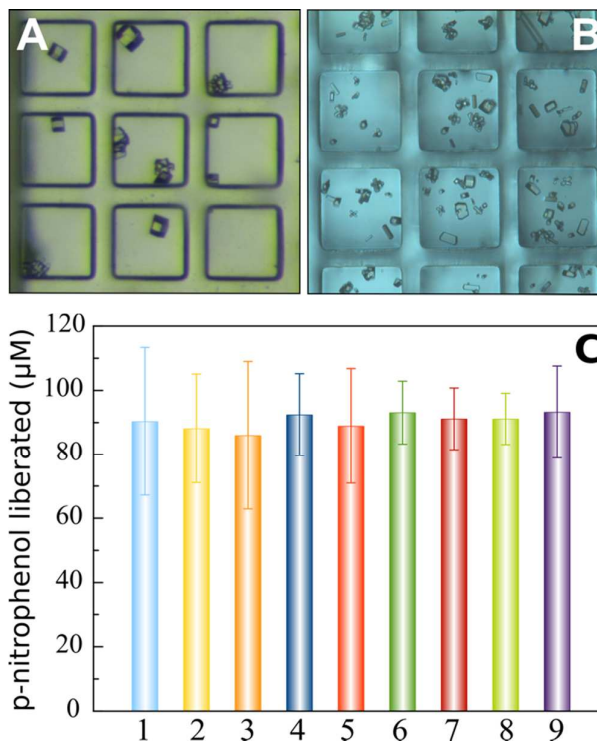
## Results and discussion

The McCLECs here presented were specifically designed to allow preferential crystallization into a micro-well array, while allowing the mixture and flow of a solution along a microfluidic channel that connects the array. This makes possible the subsequent enzymatic reaction and the recovery of the product if required. Although the experiments here presented were carried out by batch method, a continuous solution injection would transform McCLECs batch reactors in continuous flow reactors for the production of a desired product. Additionally it is worth mentioning that coupling an appropriated detection system to the McCLECs would lead to the creation of a biosensor with the possibility of operating in continuous mode. Although related optofluidic enzymatic biosensors have been previously reported<sup>24</sup> the combination of McCLECs with the Multiple Path Photonic Lab on a chip

concept<sup>48</sup> for the creation of a continuous photonic sensing platform is yet to be reported and represent a substantial advance, since it provides with unmatched advantages in terms of robustness, sensitivity and stability. The validation and proof of feasibility for these biotechnological reactor/sensor approaches are presented below.

**McCLEC validation.** In order to prove the McCLEC concept, lysozyme was selected since it is a highly characterized enzyme from both the crystallization and enzymatic points of view.<sup>49-52</sup> Tetragonal lysozyme crystals were grown in McCLEC microwells using the batch method. Crystals appeared within 8-12 h at 293 K (Fig. 3A) and, after equilibration (2-4 days) they were cross-linked (Fig. 3B), washed and dried. The devices were stored at RT for more than one year without noticeable loss of activity (see below).

Since the microchip was not conceived for being able to measure the total amount of protein crystallized, several McCLCs (Cross-linked Lysozyme Crystals in McCLEC) were prepared (chips 1 to 3) and employed to study the three main variables to carry on the enzymatic reaction, i.e. substrate concentration, temperature and reaction time. Two substrate concentrations ( $0.5 \text{ mg}\cdot\text{ml}^{-1}$  and  $1 \text{ mg}\cdot\text{ml}^{-1}$ ), three temperatures (293 K, 315 K and 325 K) and three reaction times (2 h, 3.5 h and 12 h) were tested and the extension of the reaction followed by the production of p-nitrophenol. Optimal operational conditions (>95% substrate conversion within 2 h) were achieved at 325 K using  $1 \text{ mg}\cdot\text{ml}^{-1}$  substrate.



**Fig. 3.** Lysozyme crystallization, cross-linking and CLLCs reutilization in microfluidic chips. A) Lysozyme crystals grown in 8-12 h at 293 K inside McCLEC microwells. Crystallization experiments were setup by batch method with a supersaturated protein solution at 20 mg·ml<sup>-1</sup> and 5% NaCl as precipitant agent. B) CLLCs produced by incubation two hours with 1% glutaraldehyde at RT. D) Results of enzymatic assays with 9 different McCLLCs. Each coloured bar represents the average of the total re-uses of independent McCLLCs, with its standard deviation. Chips 1-6 had been stored during 8 months dried at room temperature until use for enzymatic assays and devices 7-9 one year after the initial proofs.

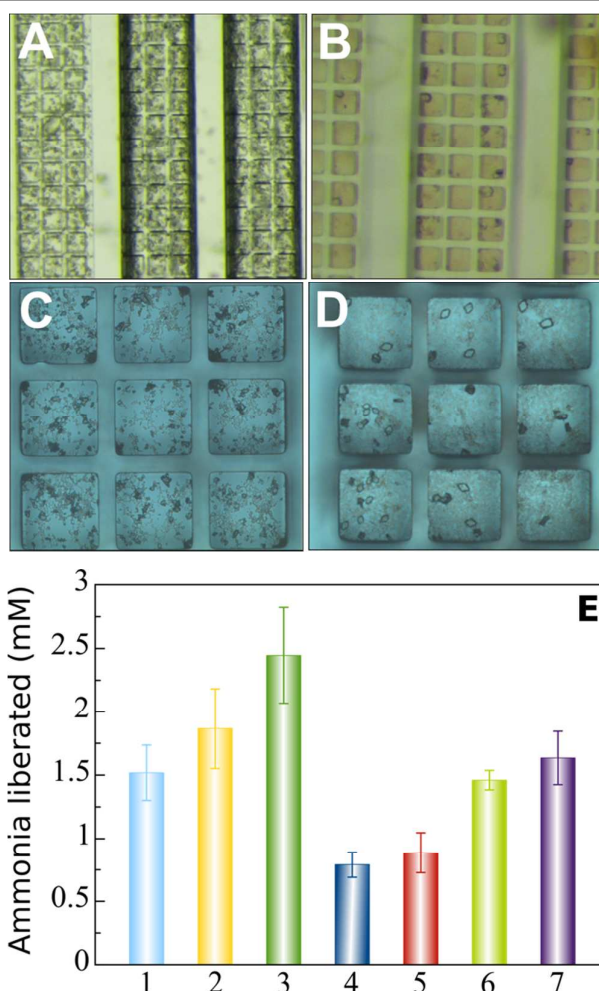
The robustness of the McCLLCs was evaluated by reutilization of different chips (once a day) by mean of two approaches: *i*) after shelf-storage at RT for 1 year (chips 1-3) and *ii*) with freshly-prepared devices (chips 4-9) (Fig. 3C). Both shelf-stored and freshly-prepared CLLCs devices were fully operative, with no detectable loss of activity after twenty re-uses (see also Fig. S5A of the supporting information). Observed deviations may be attributed to either the preparation of the substrate or the recovery and determination of the product concentration. Both experiments validate the McCLECs as a cost-effective preparation of a novel type of auto-supported enzymatic system.

**McCLECs application.** To address the applicability of the microfluidic devices in a potential biotechnological application, formamidase (FASE) was chosen for setting-up McCLFCs (Cross-linked FASE Crystals in McCLEC). FASE is a member of the nitrilase superfamily, a group of enzymes playing a key role in chemical, pharmaceutical engineering and bioremediation.<sup>53</sup> Despite the natural function of FASE is the hydrolysis of formamide, this enzyme also shows acyl-transferase activity, allowing the production of acetohydroxamic acid (lithostat®),<sup>54</sup> a potent inhibitor of urease, indicated in patients with chronic urea-splitting urinary infection.

FASE crystallization conditions used in McCLFCs were determined from batch experiments at 293 K (5 mg·ml<sup>-1</sup> of protein and 10% PEG 400, 7.5% PEG 4000, 5% PEG 8000 100 mM sodium acetate pH 4.0 as precipitant). Under these conditions, FASE crystals were obtained in 24 h. In this case, FASE crystals number and size were more disperse than in the case of tetragonal lysozyme (Fig. 4A-D).

Three different sets of McCLFCs (devices 1-3, 4 and 5-6) were independently prepared to study their reuse capability. Considering an effective trapped solution volume of 4 µL and a protein concentration of 5 mg·ml<sup>-1</sup> the maximum amount of protein that could be precipitated in each McCLFC was of ~ 20 µg. Devices 1-3 (first set) were initially used to optimize the operational conditions while keeping constant the substrate concentration at 6 mM formamide. The best reproducible results were obtained with 2 h of substrate incubation at 313 K. Following a similar protocol than with CLLC, devices 1-3 were thoroughly washed and stored at RT for eight months before the reuse experiments while the other two sets were freshly prepared. From the three devices used to prepare the second set only in one of the devices obtained crystals remained stable after the crosslinking. Therefore a third set of

McCLFC was prepared from a new purification preparation. The reuse activity of the three sets was determined during 17 cycles (1-3), 23 cycles (4) and 21 cycles (6-7). No difference in the morphology of the crystals was observed after the reuses (Fig. 4C and D). As it can be observed in Fig. 4E, device 4 showed lower activity level than the other two sets. These differences, which should not surprise when working with different protein preparations, arise as the main cause on the inter-reproducibility of the measured activity (see also Fig. S5B of the supporting information). Further visual inspection of the McCLFCs indicates, as expected, that the number and shape of FASE crystals in each set were different and therefore could be directly related to catalyst effective concentration translated as a different level of activity. On the other hand their stability along reaction cycles and storage does not seem to be affected.



**Fig. 4.** FASE crystallization, cross-linking and enzymatic assays in microfluidic devices. A) FASE crystals grown in 24 h at 293K by batch method in McCLECs with a supersaturated protein solution at 5 mg·ml<sup>-1</sup> and a polyethylenglycol mixture (20% PEG 400, 15% PEG 4000, 10% PEG 8000) in 100 mM sodium acetate pH 4.0 as crystallizing agent. B)

McCLFCs produced by incubation of the crystals for 1h at room temperature with 1% glutaraldehyde. C) and D) Photographs of CLFCs after twenty enzymatic reuses. E) Results of enzymatic assays with 6 different McCLFCs. Each coloured bar represents an enzymatic re-use of the McCLFCs in each device. Devices 1-4 had been stored during 8 months dried at room temperature until use for enzymatic assays and devices 5-6, 3 months after the initial proofs.

## Conclusions

In conclusion, a microfluidic system (McCLEC) is here proposed as a simple and unique platform for *in situ* protein crystallization, cross-linked enzyme crystals production and enzymatic catalytic bioreactions performance. The McCLEC concept has been validated with two different proteins: a commercial protein, lysozyme, which is easy to crystallize and well characterized, and a protein of pharmaceutical interest, a formamidase, produced and purified for this study. CLECs of both proteins were obtained inside McCLECs and their application as biocatalyst was studied. Reuse of CLECs has been shown possible, thus decreasing the economic costs of the enzymatic reaction, presenting McCLECs as robust and cost-effective technology. Furthermore, the McCLECs have shown excellent long-term shelf life and storage capabilities at room temperature (up to one year), not only freshly stored but also stored after several reaction cycles, without observing a decrease of the enzymatic activity. Therefore McCLECs offer a stable, robust and durable platform for enzymatic catalysis, which can be used not only for enzyme characterization and high value product synthesis, but also as a sensing platform when coupled to a detection system.

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