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Electronic Supplementary Information for:

"Compartmentalized Cross-linked Enzymatic nano-Aggregates (*c*-CLE*n*A) for Efficient In-flow Biocatalysis"

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1. Materials and Methods

All chemicals and enzymes were purchased from Sigma-Aldrich and used as received, unless otherwise stated. For the synthesis of the block copolymer, CuBr was activated using acetic acid for 3 h and dried in vacuum. Ultra-pure MilliQ water (Labconco Water Pro PS purification system) was used for the self-assembly of polymersomes and dialysis. The dialysis membranes (MWCO 12-14 kDa Spectra/Por®), Amicon Ultra- 0.5 mL centrifugal filter Unit 3 kDa (Millipore), and Ultrafree-MC centrifugal filters (with 0.1 and 0.22 µm pore size) (Millipore) were used to remove the excess of enzyme after encapsulation, and to wash the nanoreactors after the flow

experiments. The proteins used for the experiments included *Candida antarctica* Lipase B recombinant from *Aspergillus Oryzae* (CalB, E.C. 3.1.1.3.) as lyophilized powder with ~ 9 U mg^{-1} activity , esterase from porcine liver (PLE, E.C. 3.1.1.1.) as lyophilized powder with ≥ 15 U mg^{-1} activity, glucose oxidase from *Aspergillus Niger* Type II (GOx, E.C. 1.1.3.4) as lyophilized powder with 228.25 U mg^{-1} activity, and peroxidase from horseradish Type I (HRP, E.C. 1.11.1.7) with 50-150 U mg^{-1} activity. The glutaraldehyde solution (25% w/w in H_2O) was purchased from Sigma Aldrich and solutions at different concentrations were prepared for the *c*-CLE*n*A formation. Genipin (≥98% (HPLC) in powder) was also purchased from Sigma Aldrich and solutions at different concentration were prepared in MiliQ water.

Proton nuclear magnetic resonance (^{1}H NMR): ^{1}H NMR spectra were recorded on a Varian Inova 400 spectrometer with $CDCl_3$ as a solvent and TMS as internal standard. ^{1}H NMR spectra were used to determine the molecular weight of the synthesized copolymers.

Gel permeation chromatography (GPC): The dispersity (Đ) of the copolymers was determined using a Shimadzu Prominence GPC system equipped with a PL gel 5 µm mixed D column (Polymer Laboratories) and differential refractive index and UV (254 nm) detectors. THF was used as an eluent at a flow rate of 1 mL min^{-1}.

Size Exclusion Chromatography (SEC): For an efficient separation of the stomatocytes from the unencapsulated enzymes, a Shimadzu Promience SEC system equipped with a Superose™ 6 column and a UV detector (220 nm) was used. The separation was performed using filtered PBS buffer at 0.8 mL min^{-1}.

Transmission electron microscopy (TEM): TEM images were recorded using a FEI Tecnai 20 (type Sphera) at 200 kV. 5 µL sample was dropped on top of a carbon-coated copper grid *(*200 mesh, EM science*)*, and the samples were left to dry at room temperature overnight.

Scanning electron microscopy (SEM): SEM images were obtained using a Quanta 3D FEG (FEI, The Netherlands) with a field emission electron gun at 10 kV-15 kV. All samples were diluted ten times with MilliQ, and 5 µL diluted solution was placed on a silicon wafer, which was previously washed in 70% EtOH and dried at RT overnight. Prior to measurement, all samples that were drop cast on the silicon wafer were coated with gold via sputtering for 30 s at 60 mA using an EMITECH 575K coater.

Asymmetric flow field flow fractionation – light scattering (AF4-LS): The AF4-LS experiments were performed on a Wyatt Eclipse AF4 instrument connected to Shimadzu LC-20A

Prominence system with Shimadzu CTO20A injector. The AF4 was further connected to the following detectors: a Shimadzu SPD20A UV detector, a Wyatt DAWN HELEOS II light scattering detectors (MALS) installed at different angles (12.9 º, 20.6 º, 29.6 º, 37.4 º, 44.8 º, 53.0 º, 61.1 º, 70.1 º, 80.1º, 90.0 º, 99.9 º, 109.9 º, 120.1 º, 130.5 º, 149.1 º, and 157.8 º) using laser operating at 664.5 nm, a Wyatt Optilab Rex refractive index detector and a Quels detector installed at angle of 140.1 º. The detectors were normalized using bovine serum albumin protein. The AF4 channel was pre-washed with a running solution of PBS, which was also used for the separation. The processing and analysis of the LS data, and hydrodynamic radii calculations, were performed using the Astra 7.1.2 software. All AF4 separations were performed on an AF4 short channel equipped with regenerated cellulose (RC) 10 kDa membrane (Millipore) and spacer of 350 µm. The method for the AF4 fractionation is described in Table S1.

Dynamic light scattering (DLS): DLS measurements were performed on a Malvern instrument Zetasizer (model Nano ZSP). Zetasizer software was used to process and analyse the data. The results are given as an average of six runs.

Fluorescence measurements: The fluorescence measurements were performed using 96-black well

Flat Bottom microplates (Greiner Bio-One) and a Tecan Spark 10M Multidetection Microplate Reader equipped with a 530 nm excitation filter and a 590 nm emission filt, bandwidth 20 nm, integration time 40 µs.

Hydrolysis assays: All assays were performed in 96-transparent well Flat Bottom microplates (Grenier Bio-One) on a Synergy2, Biotek, Winooski, US Multidetection platereader. Reader was set at a fixed wavelength (405 nm).

In-flow setup: A tubular reactor with a filter module: ID (mm) 0.5, pore size 10 kDa, EL (cm) 20.0, TL(cm) 23.0, surface area 28.0 cm^2 was purchased from Spectrum Lab ®. The inlet was equipped with a peek ferrule (VWR©) to facilitate the syringe attachment to the tubular module.

High Performance Liquid Chromatography (HPLC): The product collected at the reactor outlet was quantified with a Shimadzu LC-20AD Prominence system, which was equipped with a RP Alltima C18 5u (150mm x 3.2mm) column and a UV detector. The column was pre-equilibrated with a solution of 0.1 % formic acid (v/v) in water and 60% acetonitrile (ACN) (v/v) was used as eluent at a flow rate of 0.5 mL min^{-1}.

Bradford assay: The Bradford method was used to quantify the enzyme loading. Pierce™ Coomassie Plus (Bradford) Assay Kit was used as described in the protocol of the assay.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): The samples of interest were mixed with 4X non-reducing loading buffer and were loaded on 4-20% Mini-PROTEAN® TGX™ Precast Gels (Biorad) according to manufacturer's instructions. The gel was stained for proteins using Pierce™ Silver Stain Kit (Thermo Fischer) for the CalB samples, and Coomassie Blue (BioRad) in all the other cases.

2. Experimental procedures

2.1. Synthesis of poly(ethylene glycol)$_{44}$-polystyrene (PEG-*b*-PS)$_{140}$ block copolymer

PEG-*b*-PS was synthesized using atom-transfer controlled radical polymerization (ATRP), according to previously reported literature procedures.[1] For the macro initiator synthesis, poly(ethylene glycol) methyl ether (5.0 g, 2.5 mmol), was twice dried by co-evaporation with toluene. In a flame-dried Schlenk tube, the poly(ethylene glycol) methyl ether was then dissolved in dry THF (2.0 mL) and triethylamine (1.04 mL, 7.5 mmol) was added to the solution. The Schlenk tube was placed on an ice bath, followed by the dropwise addition of α-bromoisobutyryl bromide (616.0 µL, 5.0 mmol) while stirring. The solution was then stirred for a further 24 h, while slowly warming to room temperature, to form a white solid due to the amine salt in the poly(ethylene glycol)$_{44}$ methyl ether 2-bromoisobutyrate colorless solution. The amine salt was filtered off and the solution was concentrated in vacuum. The precipitation of macro-initiator poly(ethylene glycol)$_{44}$ methyl ether 2-bromoisobutyrate was induced by ice-cold diethyl ether.

For the PEG$_{44}$-*b*-PS$_{140}$ synthesis, copper bromide (CuBr) (45.0 mg, 0.32 mmol) was first added to a flame dried Schlenk tube equipped with a stirring bar under argon atmosphere. The Schlenk tube was sealed with a septum, and evacuated for 15 min, after which argon was filled back into the flask. PMDETA (66.0 µL, 0.32 mmol) was dissolved in 0.5 mL toluene and added to the CuBr. The mixture was left stirring for 15 min with argon for oxygen removal. Polyethylene glycol macroinitiator (215 mg, 0.10 mmol), dissolved in 1 mL toluene, was added into the Schlenk tube. The solution was degassed for 15 min while cooling in an ice bath. Distilled styrene (5.0 ml, 43.6 mmol) was added to the reaction mixture. The mixture was then degassed and the Schlenk tube was inserted into a preheated 70 ^{o}C oil bath, overnight. At the end of the reaction, dichloromethane (CH_2Cl_2) (75 mL) was added to the polymer solution and the mixture was filtered over an alumina column to remove the CuBr. The final solution was then concentrated and the polymer was precipitated in cold methanol, filtered and dried overnight in vacuum. The amphiphilic polymer obtained, PEG$_{44}$-b-PS$_{140}$ had a number average molecular weight (MW) of 16.8 kg mol^{-1} and Đ= 1.06.

2.2. General procedure for polymersome preparation

The polymersomes were self-assembled using a slightly modified variation of a previously reported solvent switch method. In short, 20.0 mg synthesized PEG_{44}-*b*-PS_{140} polymer was dissolved in a 1.0 mL mixture of THF: dioxane (4:1 v/v), to which 1.0 mL MiliQ was added via a syringe pump with a flow rate of 1.0 mL h^{-1}, resulting in the formation of a cloudy solution. The assembly was performed inside a 5.0 mL vial which contained a magnetic stirring bar and which was capped with a septum. The cloudy solution was then dialyzed against MiliQ water for 24 h, with the MiliQ frequently refreshed.

Stomatocyte nanoreactors were prepared using the previously reported solvent addition methodology.[2] 300 µL THF:dioxane solution (4:1 v/v) was added via a syringe pump at a rate of 300 µL h^{-1} to 500 µL of the previously prepared polymersome solution (10.0 mg mL^{-1}), while continuously stirring. The organic mixture was removed from the polymeric solution using spin filtration (20 mins, 13523 rcf) which was repeated two times using Amicon 3 kDa filters. The polymersomes were re-suspended to their initial concentration by adding MilliQ water. At the end of this process, opened neck stomatocytes (Figures S1, S2) were formed which were used for enzyme entrapment. Consequently, 12 mg mL^{-1} CalB in a 50 mM sodium phosphate buffer, pH 7.5, was added to the stomatocytes and mixed vigorously at 7000 rpm for 30 mins. To narrow the neck of the stomatocytes (Figures S1, S2), 150 µL THF: dioxane (4 : 1 v/v) at 150 µL h^{-1} flow rate was added to the solution. To remove non-encapsulated enzymes, stomatocytes were purified from the solution mixture using size exclusion chromatography (SEC). After SEC, the stomatocytes were concentrated again in a final volume of 500 µL (10 mg mL^{-1}).

For the preparation of CalB and PLE samples, the initial concentration of each enzyme was varied between 3.0 and 16.0 mg mL^{-1}. In the case of the GOx/HRP loaded stomatocytes, the molar ratio between GOx:HRP was kept at 1:4 (mol/mol), with [GOx] = 4.05 mg mL^{-1} and [HRP] = 4.77 mg mL^{-1} and with an encapsulation efficiency of 13%.

2.3. General procedure for the formation of compartmentalized cross-linked enzyme nano aggregates (*c*-CLE*n*A) with Glutaraldehyde

Having ensured complete removal of free enzyme from the previously prepared stomatocyte nanoreactors, glutaraldehyde (100 µL, at different concentrations varying between 30 mM and 150 mM) was slowly added, at a rate of 100 µL h^{-1}, to a solution of enzyme loaded stomatocytes while stirring. In case of either CalB or PLE, the cross-linking reaction was quenched with 1mL of sodium phosphate buffer (1M, pH = 7.5) solution, and in the case of GOx/HRP, the reaction was quenched with 1mL of PBS (pH = 7.4). To remove the excess of

buffer and glutaraldehyde, all resulting *c*-CLE*n*As were concentrated via spin filtration. In the case of CalB and PLE, the *c*-CLE*n*As were re-dispersed in sodium phosphate buffer (50 mM, pH = 7.5). In the case of GOx/HRP, the *c*-CLE*n*A was re-dispersed in PBS (pH = 7.4). In all cases, no change of the stomatocytes' morphology was observed after glutaraldehyde addition (Figure S2 and S15).

2.4. General procedure for the formation of cross-linked enzyme aggregates (CLEA) with Glutaraldehyde

To a solution of CalB (3 mg mL^{-1}, in sodium phosphate buffer pH 7.5), 100 µL of glutaraldehyde (150 mM) was slowly added, at a rate of 100 µL h^{-1} while stirring. The reaction mixture was incubated for 8 h. To remove the excess of buffer and glutaraldehyde, all resulting CLEAs were concentrated via spin filtration (0.1 µm), and imaged using SEM (Figure S6)[3].

2.5. General procedure for the formation of compartmentalized cross-linked enzyme nano aggregates (*c*-CLE*n*A) with Genipin

1 mL of genipin solution (concentration 30mM to 50 mM) was added to 100 µL of stomatocyte sample in an Eppendorf. The solutions were kept for 24h at RT under gentle stirring.[4] For the PLE *c*-CLE*n*A formation, stomatocyte samples were prepared at a concentration of 8mg mL^{-1} which resulted in 17% of encapsulation. For the GOx/HRP *c*-CLE*n*A, the molar ratio between GOx:HRP was kept at 1:4 (mol/mol), with [GOx] = 4.05 mg mL^{-1} and [HRP] = 4.77 mg mL^{-1} and the encapsulation efficiency was 13%.

After 24h Genipin was removed from the solution via centrifugation, using10 kDa filters, at 12000 rpm for 15 min. The occurrence of *nano*-aggregates formation was also confirmed by the dark colour of the solution. Finally the *c*-CLE*n*A were recollected from the filter and their volume adjusted to 100 µL.

2.6. General procedure for the formation of cross-linked enzyme aggregates (CLEA) with Genipin

To a solution of PLE (3 mg mL^{-1}, in sodium phosphate buffer pH 7.5), 1 mL of genipin (50 mM) was added while gently stirring the solution at RT. The reaction mixture was incubated for 24h. The solution became dark after the CLEA formation. To remove the excess of buffer and genipin, all resulting CLEAs were concentrated via spin filtration (0.1 µm), and imaged using SEM (Figure S17).

2.7. Quantification of enzyme loading

The Bradford assay was used to quantify the amount of enzyme loaded in the stomatocytes and in the *c*-CLE*n*As. All the samples were treated with CH_2Cl_2 to completely dissolve and remove the polymeric membrane, which would alter the absorbance measured in the test. 150 µL of enzyme loaded stomatocytes were mixed with 500 µL of CH_2Cl_2 for 30 mins. . The final solution was then spin filtered with a centrifugal filter Unit 3 kDa (Millipore) to remove the organic solvent. The fraction collected was adjusted with buffer to the final volume of 150 µL. The measurements were performed in triplicate using 50 µL. The encapsulation efficiency was also determined by measuring the absorbance of empty stomatocytes and subtracting it from the measured absorbance of the enzyme-loaded stomatocytes. In both cases, similar encapsulation efficiencies were obtained. For protein quantification, the Coomassie Plus (Bradford) Assay Kit was used (Pierce™) according to manufacturer's instructions. In each cuvette both 1.5 mL of Coomassie reagent and 50 µL of sample were added. Before measuring the absorbance at 595 nm, all samples and the standard solutions were incubated for 5 mins at room temperature and the spectrophotometer was calibrated with a cuvette containing a blank solution.

Using the protein concentrations that were measured, the encapsulation efficiency (e.e. %) was determined by considering the protein concentration in the initial feed solution.

$$\text{e.e.}(\%) = \frac{\text{Protein concentration measured with the Bradford assay (mg mL}}{\text{Lowest value of protein concentration used in the feed (mg mL}} \times 100$$

The encapsulation efficiency is given as average of triplicate measurements per each sample (Table S2). These encapsulation efficiencies were measured before the *c*-CLE*n*A formation.

2.8. SDS-PAGE analysis

The effectiveness of enzyme cross-linking for CalB was analyzed using SDS-PAGE. Different sample volumes of the free and cross-linked enzymes were mixed with 4X non-reducing loading buffer and were loaded, along with the marker for protein molecular weight standards (Precision Plus Protein™ All Blue, Biorad) on 4-20% Mini-PROTEAN® TGX™ precast gels

(Promega). As per manufacturer's instructions, the electrophoresis was carried out at constant voltage (110V) for 2h and the gel was stained using Pierce™ Silver Stain Kit, ThermoFischer; in the case of CalB, after gel fixation, the gel was sensitized for 1 min and stained for just 5-10 mins. The gel was developed for 1 min and the reaction was stopped with 10 % acetic acid. For the PLE and GOx/HRP reactions, the gel was stained with Bio-Safe™ Coomassie Stain, Biorad, for 1-4 hr and destained with MilliQ overnight. The gel was visualized by the white light box. For all the experiments the concentration of enzyme and the loaded volume was the same in each lane.

2.9. CalB and PLE activity assays

All activity assays were conducted in triplicate. The activity of both CalB and PLE was assessed using an assay which monitors the hydrolysis reaction of *p*-nitrophenyl acetate to the *p*-nitrophenol product. The product formation over time was monitored at 405 nm.

The reaction was performed in a slightly basic environment, using a filtered sodium posphate buffer (50mM, pH 7.5) at 25 °C. In all experiments, a master mix solution of 2 mM *p*-NPA in DMSO was used. The activity of free CalB, CalB loaded stomatocytes and CalB *c*-CLE*n*A was measured (Figure 3A). The same buffer and substrate conditions were applied when the activity of free PLE, PLE loaded stomatocytes and PLE *c*-CLE*n*A was assessed (Figure 3B). The reactions were carried out at different concentrations of substrate (ranging from 0 to 2 mM) and the activity was obtained by using equations 1 and 2:

(Equation 1.) $$\frac{U}{mL} = \frac{\left(\left(\frac{\Delta Abs}{min}\right) \times V_{tot} \times Dilution\right)}{\left(\varepsilon \times V_{enzyme}\right)}$$

Where $$\frac{\Delta Abs}{min} = \frac{\Delta Abs\ (reaction)}{min} - \frac{\Delta Abs\ (background)}{min}$$

V_{tot} = is the total volume in the well,

Dilution = is the dilution factor used in the enzyme solutions;

ε = 18.5 mM^{-1} cm^{-1} is the extinction coefficient of *p*-NP at 405 nm.

V_{enzyme} = volume of *c*-CLE*n*As, enzyme loaded stomatocytes or free enzymes used.

$$\frac{U}{mg} = \frac{\frac{U}{mL}}{\frac{mg}{mL}(enzyme)}$$

(Equation 2.)

The absorption (Abs (background)) is given by the absorbance of the negative control (the free enzymes, the enzyme loaded stomatocytes and the *c*-CLE*n*As absorbance without substrate) together with the absorbance due to self-hydrolysis of substrate (*p*-NPA in buffer without enzyme).

2.10. GOx/HRP activity assays

The activity of the GOx/HRP *c*-CLE*n*A was studied using the cascade reaction between HRP and GOx, where (D)-glucose and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® red) are substrates that are converted to the products, gluconolactone and resorufin. The reaction was monitored using resorufin fluorescence (λex = 530 nm, λem = 590 nm) on the Spark ® 10M microplate reader (TECAN). The samples tested included the free enzymes, GOx/HRP loaded stomatocytes and the combined *c*-CLE*n*A samples prepared using different glutaraldehyde concentrations (30, 60, and 120 mM). 20μL sample was mixed with 130 μL reaction master mix containing Amplex red (250 μM), and (D)-glucose (final concentrations of either 1 mM, (Figure S11) or 20 mM for the *c*-CLE*n*A prepared at different amounts of glutaraldehyde (Figure 3C) at 25 °C in PBS, pH 7.4). The fluorescence values were background subtracted. Each assay was conducted twice. The error is the standard deviation from three different assays on samples at the same loading. The samples were always diluted 10 times unless stated otherwise. The final results are given as the fluorescent signal of resorufin corrected for the background.

2.11. Flow experiments

Spectrum Lab ® tubular filters were used as tubular reactors for the in-flow experiments. The flow setup (Figure S12) was equipped with two perpendicular inlets and two outlets, membrane and substrate inlets and membrane and product outlet. Membrane inlet and outlet were respectively used to load CalB (cross-linked or non-cross-linked) loaded stomatocytes on a 10 kDa membrane and recollect them after the reaction. Substrate inlet and product outlet were used to feed *p*-nitrophenyl acetate into the flow reactor and to collect the product, respectively. The tangential configuration of this flow setup does not only facilitate the recollection of the polymeric vesicles at the end of each flow run, but also ensures a good contact the stomatocytes and the substrates.

In separate experiments: 0.5 mL of CalB loaded stomatocyte solution and 0.5 mL CalB *c*-CLE*n*A solutions were introduced in the 10 kDa membrane of the reactor *via* a syringe pump (at 0.1 mL min^{-1}), ensuring homogeneous distribution throughout the whole length of the reactor's membrane. The size of the membrane (10 kDa) ensured the capture of the stomatocytes and prevented their loss. In the case of the first set of experiments in which five catalytic in-flow runs were performed, a 3 mM *p*-NPA stock solution in a mixture of 50 mM sodium phosphate buffer, pH 7.5, and 5% DMSO (v/v) was prepared, and fed to the tubular reactor using the lateral inlet via a syringe pump at 0.3 mL min^{-1}. In the case of the ten runs experiments this flow rate was increased to 0.6 mL min^{-1}. In both cases, the product was collected at the end of the reaction (after ~13 mins), from the product outlet before performing further HPLC measurements. The flow reactor was washed by flushing the membrane with MilliQ water before the next catalytic experiment.

The CalB loaded stomatocytes and CalB *c*-CLE*n*A were unloaded from the membrane and recollected at the membrane outlet by manual injection of 1 mL of MilliQ . After washing with sodium phosphate buffer (50mM, pH = 7.5) three times using spin filtration (0.22 μm Ultrafree ®-CL filters) the recovered particles were re-dispersed in the same buffer and the final volume was adjusted again to 0.5 mL and used in the next run. Both CalB loaded stomatocytes and CalB *c*-CLE*n*A retained their structural integrity after all catalytic cycles (Figure S14). The same procedure was applied for PLE *c*-CLE*n*A formed using Genipin (Figure 4).

Supplementary Figures and Tables

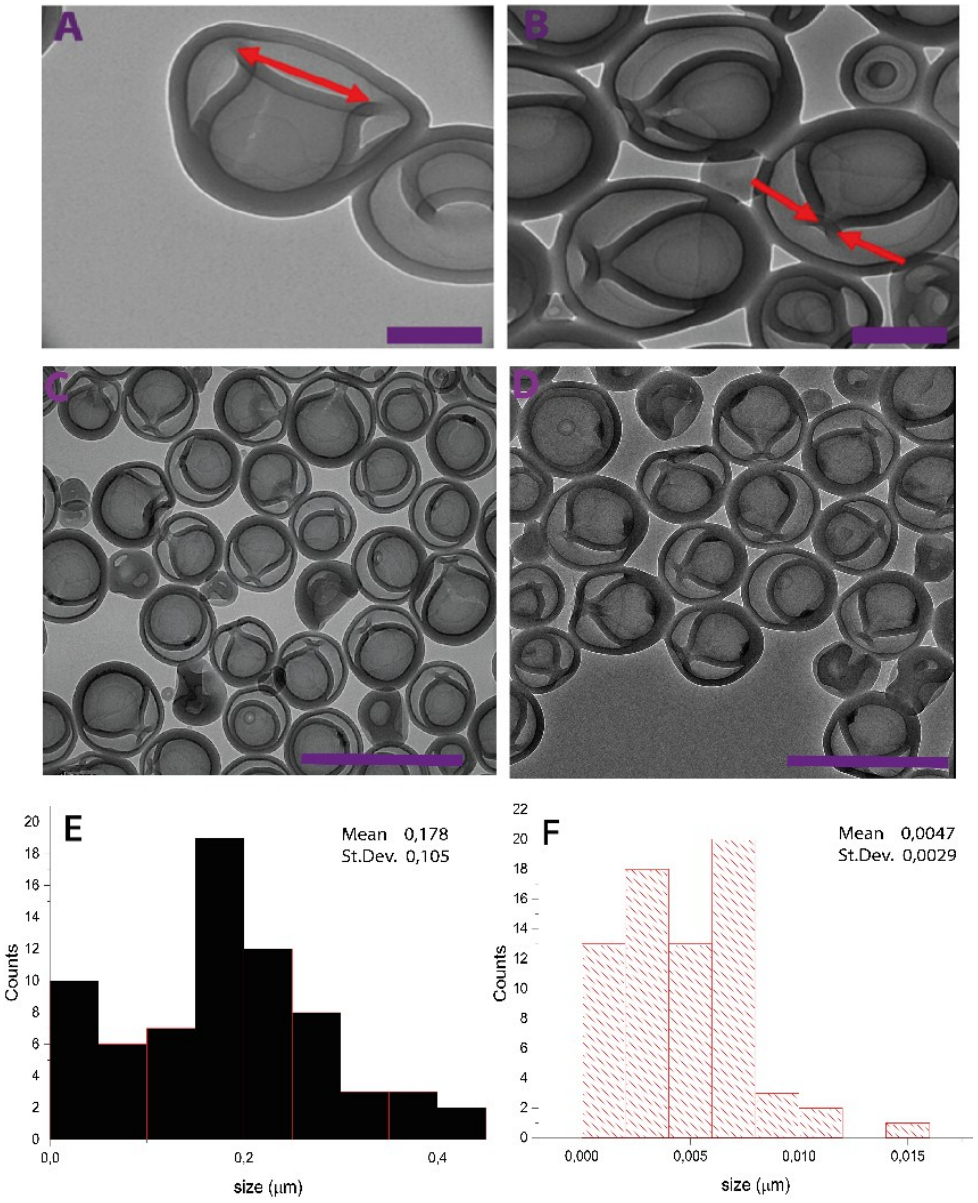

Figure S1- TEM images of A) Open neck stomatocytes B) CalB loaded stomatocytes after neck closure. C) PLE- loaded stomatocytes after neck closure. D) GOx/HRP- loaded stomatocytes after neck closure. Scale bar corresponds to 100nm in A) B) and 1 µm in C) D). Histogram analysis of stomatocytes' neck size: E) open neck F) closed-neck

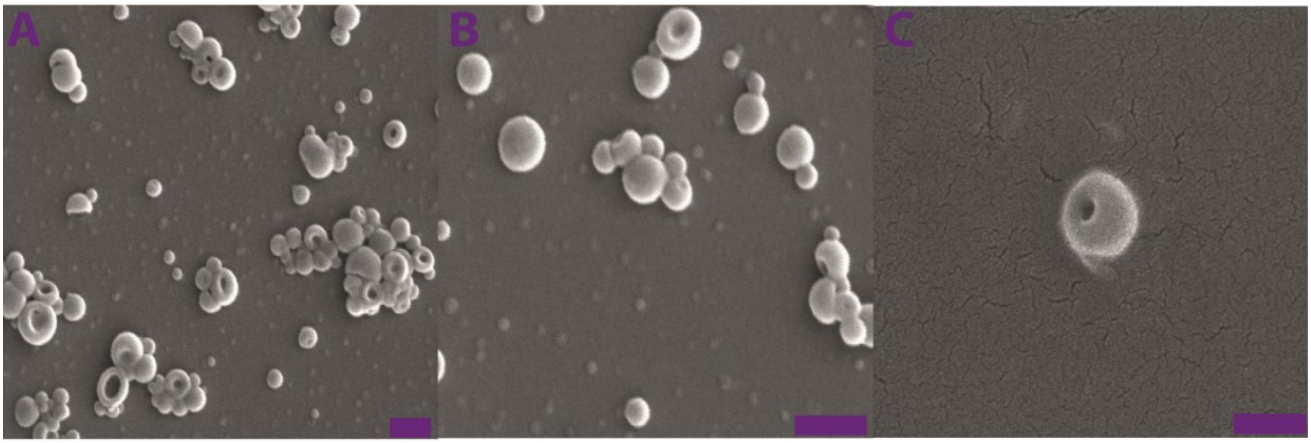

Figure S2- SEM images of A) open neck stomatocytes B) Stomatocytes after neck closure C), and subsequent cross-linking reaction using 100 mM of glutaraldehyde. Scale bars correspond to 500 nm

Start (min)	End (min)	Mode	Crossflow start (mL min^{-1})	Crossflow end (mL min^{-1})
0	1	Elution	3.00	3.00
1	2	Focus	-	
2	3	Focus + inject	-	
3	4	Focus	-	
4	6	Elution	3.00	1.17
6	8	Elution	1.17	0.49
8	10	Elution	0.49	0.24
10	13	Elution	0.24	0.10
13	30	Elution	0.10	0.10
30	31	Elution	0.00	0.00
31	32	Elution + inject	0.00	0.00
32	37	Elution	0.00	0.00

Table S1- General method for the AF4 fractionations. The flow conditions applied were the following: 1.0 mL min-1 detector flow, 1.00 mL min^{-1} focus flow and 0.20 mL min^{-1} injection flow.

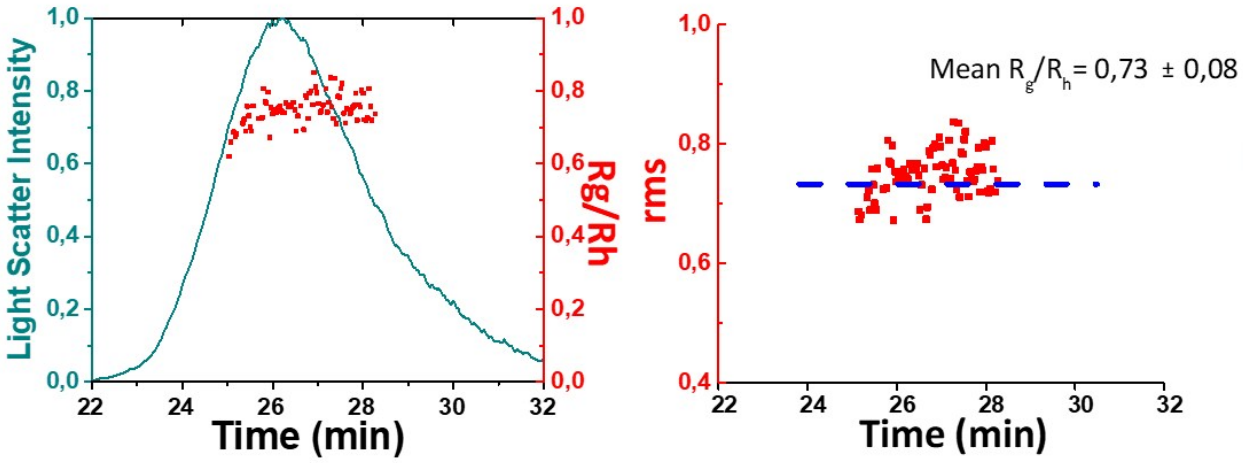

Figure S3- (Left) AF4 fractogram of CalB loaded Stomatocytes. (Right) The ratios between the radius of gyration (R_g) and the hydrodynamic radius of (R_h) of CalB loaded stomatocytes. The blue dotted line represents the mean value of these ratios.

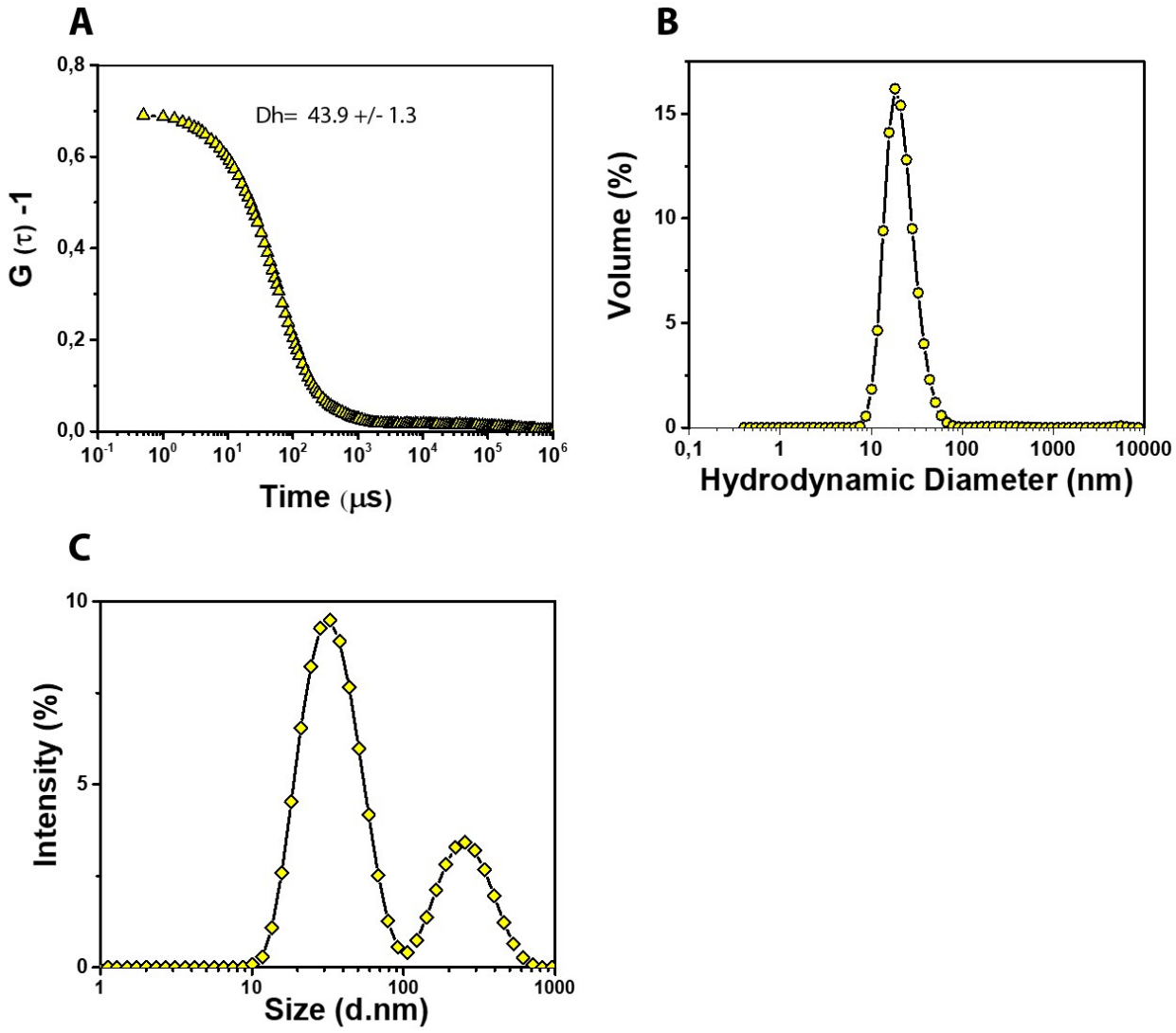

Figure S4- A) correlation function of the released CalB CLE*n*A; B) volume profile derived from A, showing a size distribution of ~43 nm and a PDI of 0.5, C) DLS Intensity distribution of the released CalB CLE*n*A. Please note the two peaks; one is corresponding to the released CLE*n*A (~43 nm) and the other is corresponding to the stomatocytes left in solution (~300nm).

CalB concentration in the intital feed solution (mg mL $^{-1}$)	**Encapsulation Efficiency (%)**
3.0	5.71 ± 0.70
6.0	8.95 ± 0.14
12.0	14.28 ± 1.72
8.0	11.93 ± 1.14

14.0	22.20 ± 0.76
16.0	32.93 ± 0.24
PLE concentration in the intital feed solution (mg mL $^{-1}$)	**Encapsulation Efficiency (%)**
3.0	4.91 ± 1.62
6.0	9.51 ± 1.14
8.0	11.5 ± 2.34
14.0	18.75± 1.46
16.0	21.03 ± 2.54

Table S2- Loading efficiency of the stomatocyte samples prepared with different enzymes. Please note that these encapsulation efficiencies are higher than statistical. This is possibly because of a templating effect mechanism as result of the clustering of enzymes inside the nanocavity of the stomatocytes [1,2].

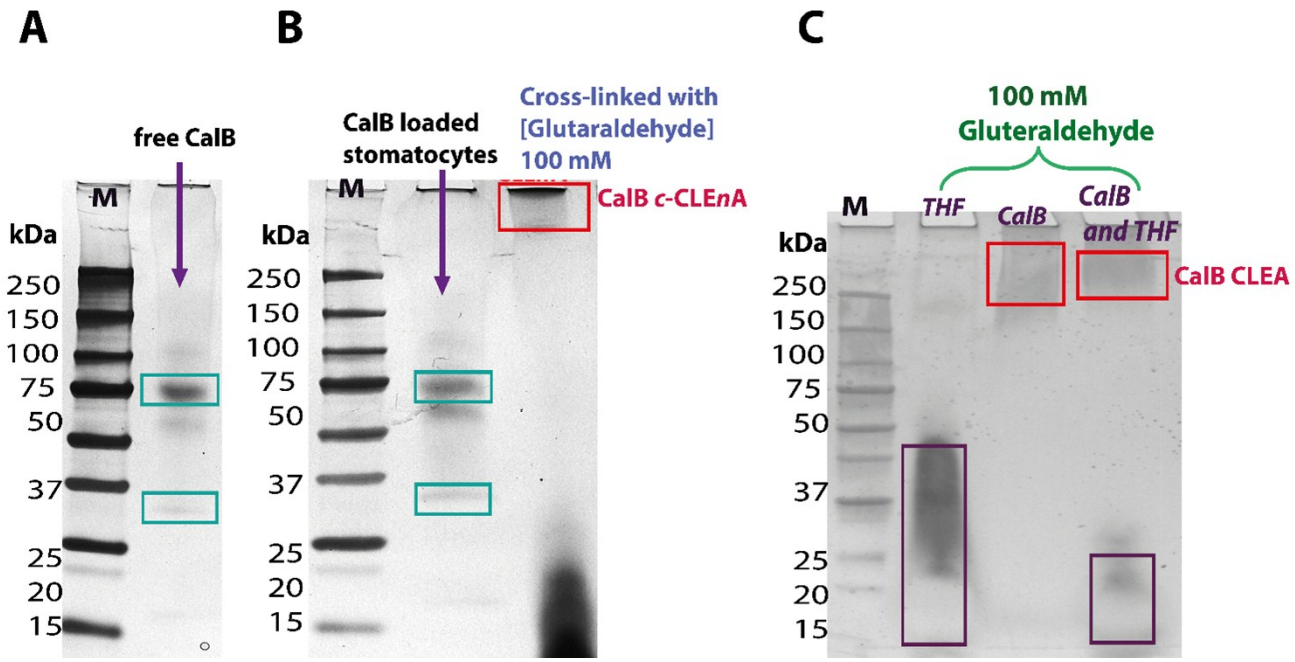

Figure S5 – A) SDS-PAGE gel analysis of free CalB. The first lane contains Precision Plus Protein™ All Blue protein molecular weight markers (M); a solution of CalB is loaded in the second lane (arrow). Both the expected molecular weight of CalB is 34 kDa and its dimer (at

~70 kDa) are visible (green rectangles). B) SDS-PAGE gel analysis of CalB c-CLE*n*A formation upon cross-linking of CalB loaded stomatocytes with 100 mM of glutaraldehyde. The first lane contains Precision Plus Protein™ All Blue protein molecular weight markers (M); CalB loaded stomatocytes are loaded in the second lane (arrow) and CalB *c*-CLE*n*A formation in the third lane. The expected molecular weight of CalB is 34 kDa, the dimeric form at ~70 kDa is also visible in the CalB loaded stomatocytes (green rectangles). The disappearance of the bands indicates *c*-CLE*n*A formation, which is also confirmed by the appearance of higher molecular weight species (red rectangle).C) SDS PAGE- Control on the low MW smear. Lane 1:Precision Plus Protein™ All Blue protein molecular weight markers (M); Lane 2: mixture of 100 mM glutaraldehyde and 150 µL of THF. Lane 3: mixture of CalB and glutaraldehyde (100 mM); lane 4: CalB *c*-CLE*n*A. The appearance of the smear at low MW only in the lanes 1 and 4 suggest that the smear is caused by presence of THF

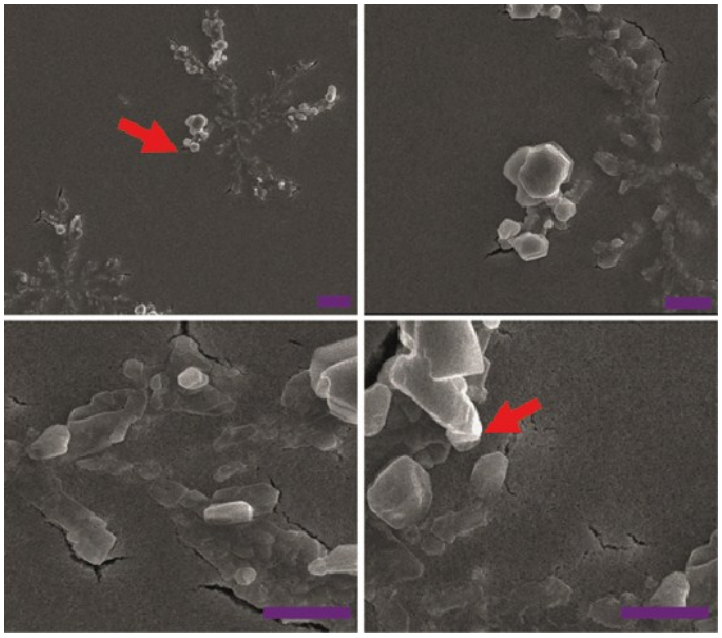

Figure S6- SEM images of non-templated CLEA formed from free CalB in solution. Please note the broad size distribution (red arrows indicate macro-cluster formation in the range of 5 to 20 µm) as a result of uncontrolled cross-linking. Scale bars correspond to 10 µm.

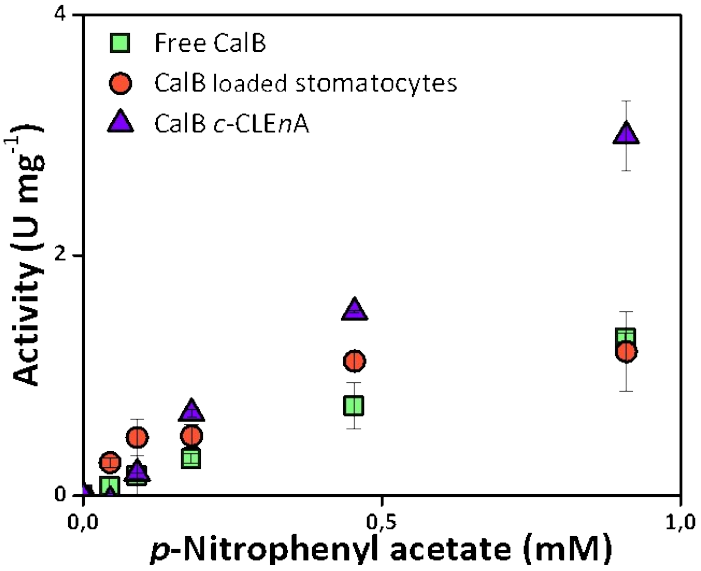

Figure S7- Comparison between specific activity (U mg^{-1}) of free CalB, CalB loaded stomatocytes (with non-cross-linked CalB), and CalB *c*-CLE*n*A. The absorbance of the product is measured at 405 nm at different concentration of substrate.

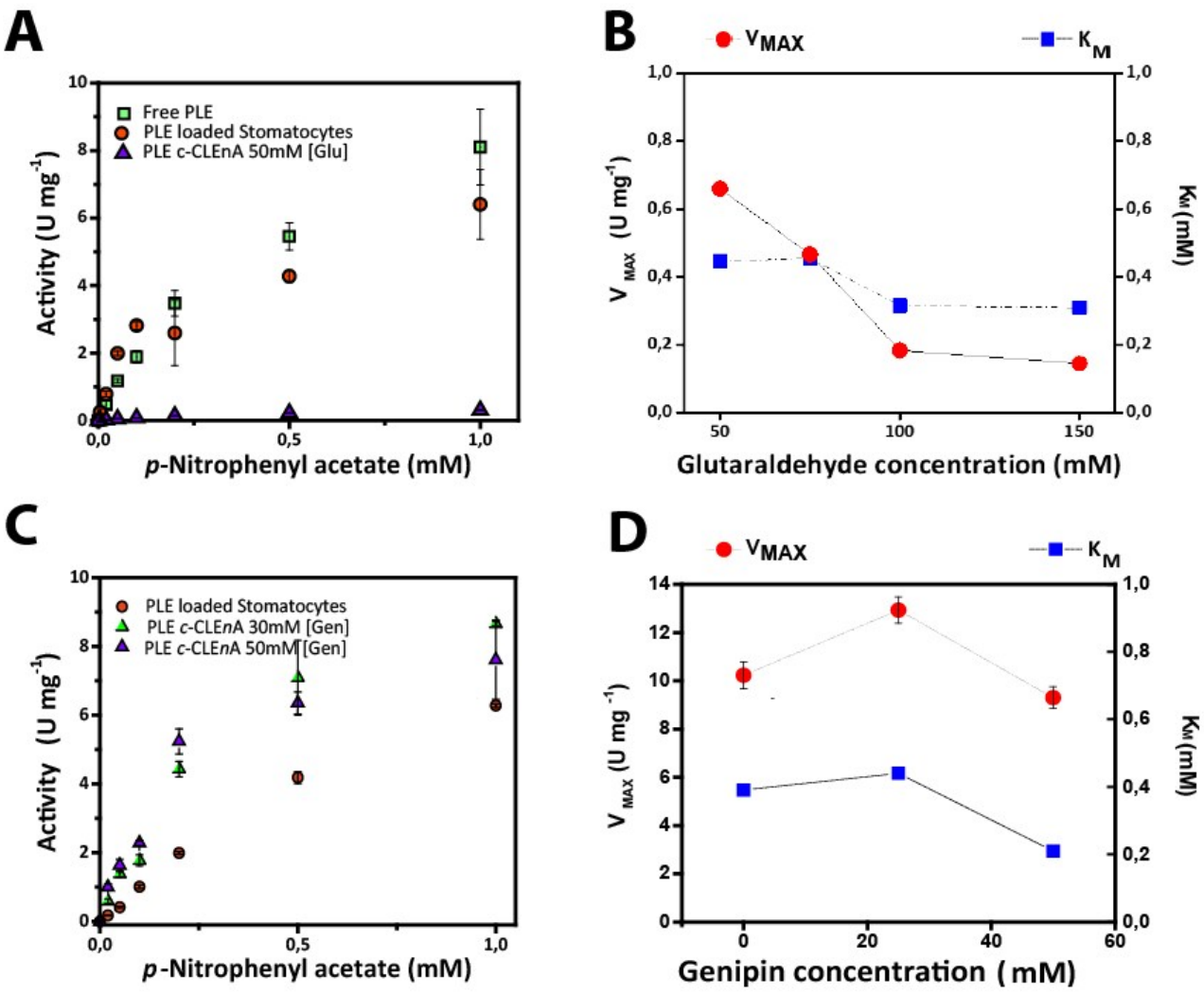

Figure S8- A) Comparison between specific activity (U mg^{-1}) of free PLE, PLE loaded stomatocytes (with non-cross-linked PLE), and PLE *c*-CLE*n*A formed with 50 mM of glutaraldehyde. The absorbance of the product is measured at 405 nm at different concentrations of substrate. B) PLE *c*-CLE*n*As prepared at different glutaraldehyde concentrations show a clear decrease of V_{MAX} with increasing concentration of glutaraldehyde. C) Comparison between specific activity (U mg^{-1}) of PLE loaded stomatocytes (with non-cross-linked PLE), and PLE *c*-CLE*n*A prepared with genipin at different concentrations. D) PLE *c*-CLE*n*As prepared at different genipin concentrations. Please note the high V_{MAX} when 30 mM of genipin was used, and the low V_{MAX} when 50 mM of genipin was used.

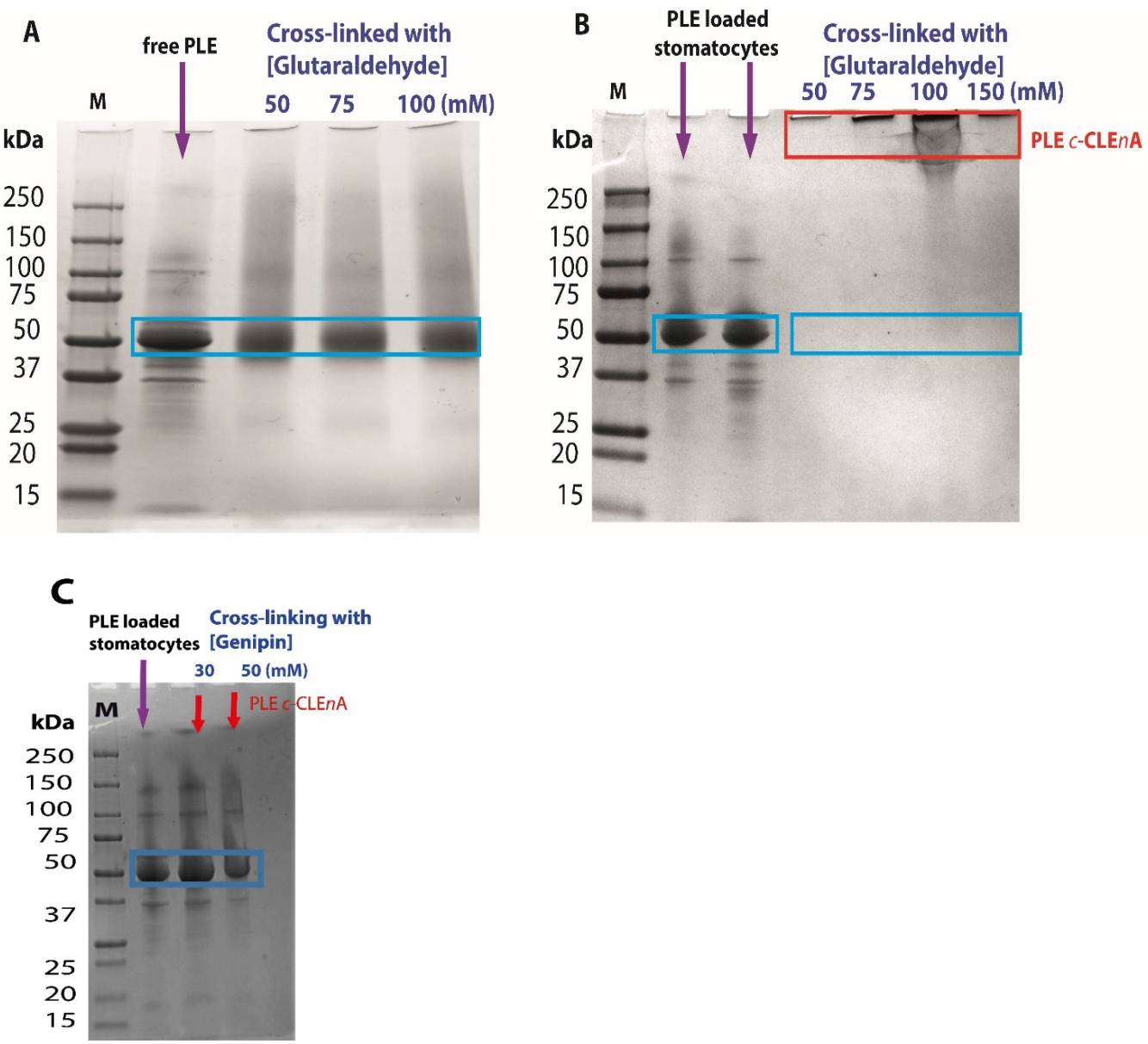

Figure S9- A) SDS-PAGE gel of PLE cross-linked with different glutaraldehyde concentrations in solution. From left to right: molecular weight ladder (M), the second lane (indicated with an arrow)

non-cross-linked free PLE enzyme. Lane 3-5: different concentrations of glutaraldehyde used to cross-link the free PLE as depicted on top of each lane (50-75-100 mM). PLE has a molecular weight of ~60kDa (blue rectangle), the band was still observed after the addition of glutaraldehyde. B) SDS-PAGE gel of PLE loaded stomatocytes cross-linked with different glutaraldehyde concentrations to form PLE *c*-CLE*n*A. From left to right: molecular weight ladder (M), lane 2 and 3 (indicated with arrows) PLE loaded stomatocytes before cross-linking. Lane 4-7: different concentrations of glutaraldehyde used for PLE *c*-CLE*n*A formation, as depicted on top of each lane (50-75-100-150 mM). The disappearance of the band at 60 kDa is indicated with the blue rectangle and the appearance of higher molecular weight species is confirmed (red rectangle). C) From left to right: molecular weight ladder (M), the second lane (indicated with the purple arrow) contains the PLE loaded stomatocytes. Lane 3-4: (indicated with red arrows) contain PLE *c*-CLE*n*A formed at different concentrations of genipin as depicted on top of each lane (30-50 mM).In this case, the cross-linking efficiencies with genipin are lower than those with glutaraldehyde, as confirmed from the intensity of the bands (blue rectangle). All the samples in each lane are at the same concentrations.

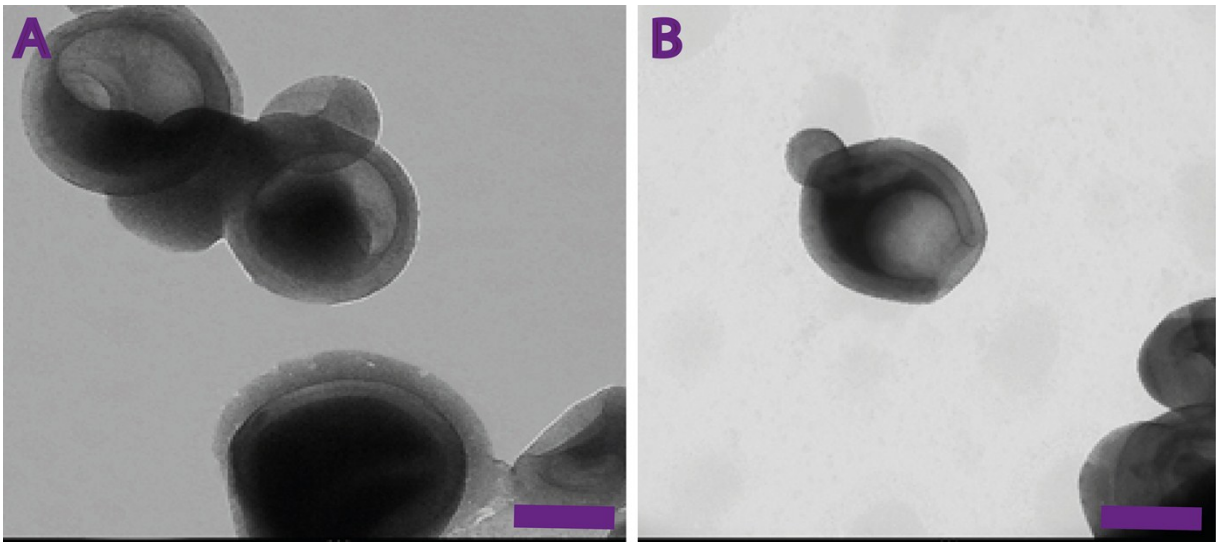

Figure S10- TEM images of A) PLE c-CLEnA formed with genipin B) GOX/HRP c-CLEnA formed with genipin. Scale bar corresponds to 100nm

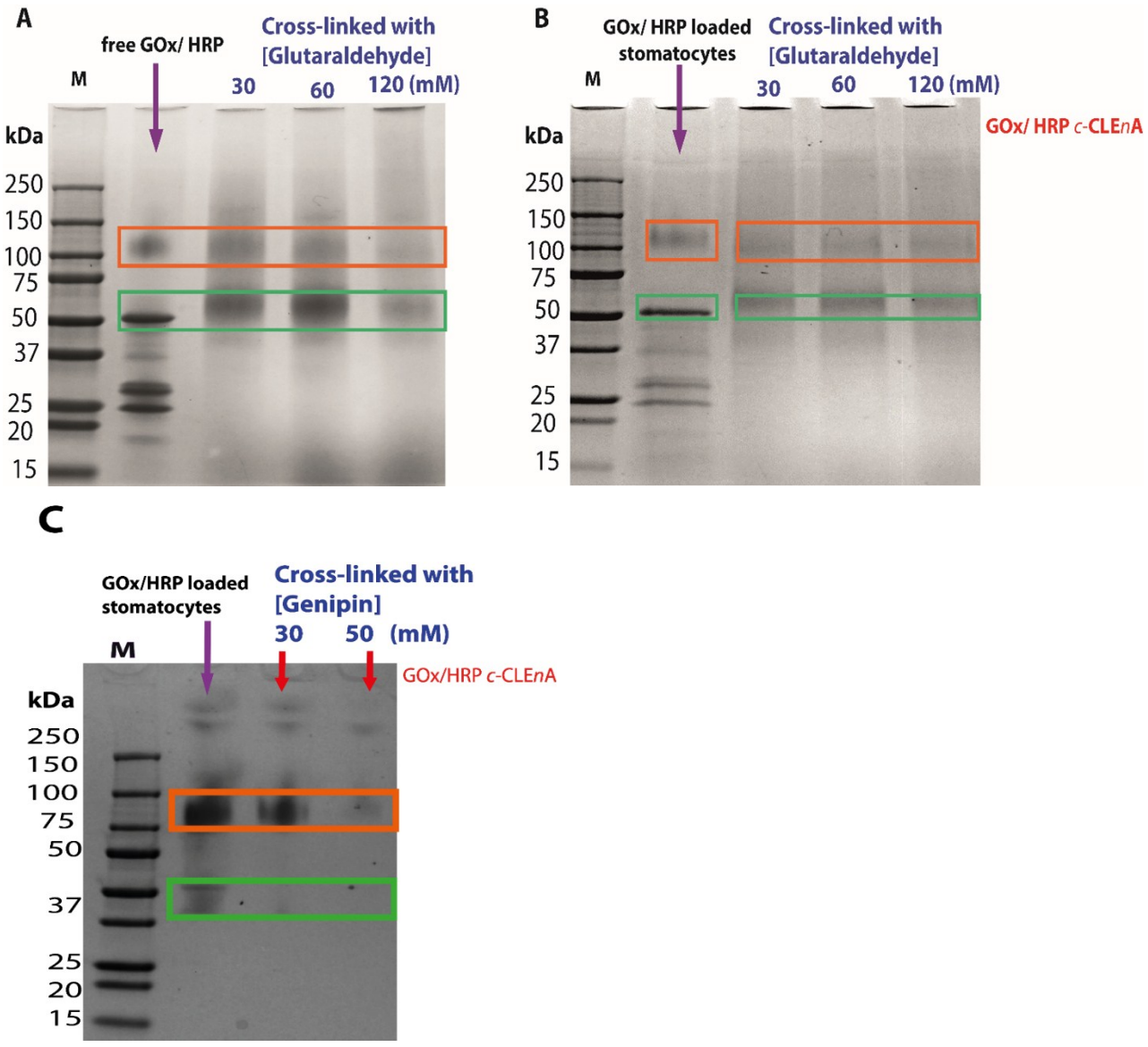

Figure S11- A) SDS-PAGE gel of free GOx/HRP cross-linked with different glutaraldehyde concentrations. From left to right: the molecular weight ladder (M), the second lane (indicated with an arrow) contains the free GOx/HRP enzymes. Lane 3-5: different concentrations of glutaraldehyde used to cross-link the free GOx/HRP as depicted on top of each lane (30-60-120 mM). The subunit enzyme molecular weights (44 kDa HRP, 160 kDa GOx) are indicated in the green and orange rectangles respectively; no disappearance of the bands is observed after the addition of glutaraldehyde. B) SDS-PAGE gel of GOx/HRP loaded stomatocytes cross-linked with different glutaraldehyde concentrations to form combined GOx/HRP *c*-

CLE*n*A. From left to right: molecular weight ladder (M), the second lane (indicated with the arrow) contains the GOx/HRP loaded stomatocytes. Lane 3-5: different concentrations of glutaraldehyde used for the combined GOx/HRP *c*-CLE*n*A as depicted on top of each lane (30-60-120 mM). The cross-linking is confirmed by the gradual disappearance of the GOx and HRP monomer bands. C) From left to right: molecular weight ladder (M), the second lane (indicated with the arrow) contains the GOx/HRP loaded stomatocytes. Lane 3-4: (indicated with the red arrows) contain GOx/HRP *c*-CLE*n*A formed at different concentrations of genipin as depicted on top of each lane (30-50 mM). All the samples in each lane are at the same concentrations.

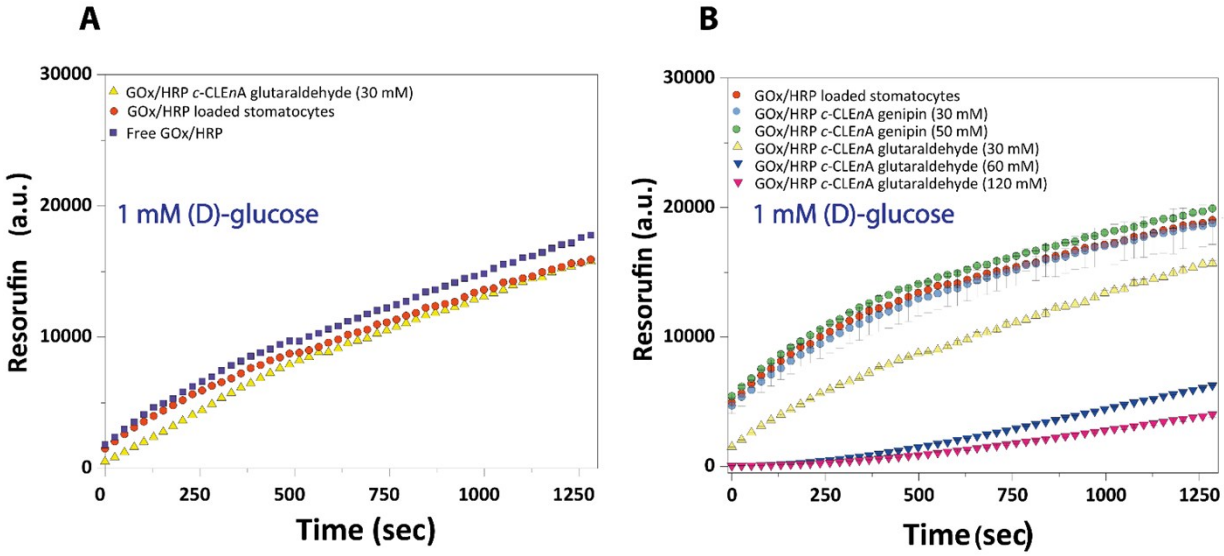

Figure S12- A) Resorufin formation at 1 mM of (D)-glucose when free GOx/HRP activity is compared with GOx/HRP loaded stomatocytes (13% loaded diluted 10 times) and GOx/HRP *c*-CLE*n*A formed with 30 mM of glutaraldehyde (13% loaded diluted 10 times). B) Resorufin formation at 1 mM of (D)-glucose when GOx/HRP loaded stomatocytes (13% loaded diluted 10 times) are compared with GOx/HRP *c*-CLE*n*A formed with 30-60-120 mM of glutaraldehyde (13% loaded diluted 10 times) and GOx/HRP *c*-CLE*n*A formed with 30-50 mM of genipin. Average results on triplicates with samples at the same loading efficacy.

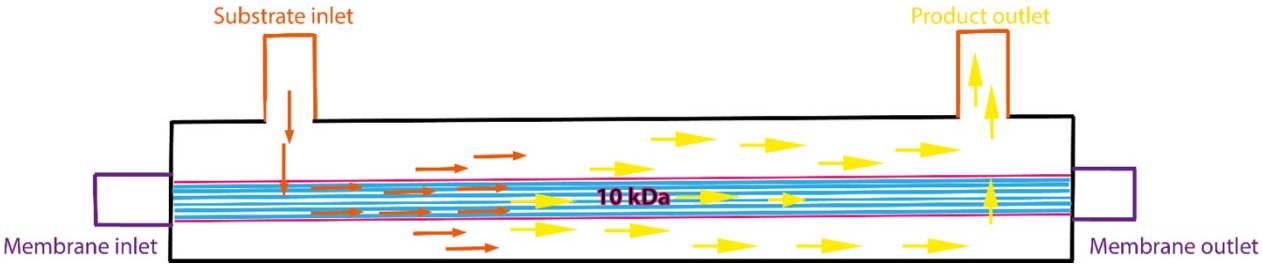

Figure S13- Schematic representation of the flow reactor system. The orange arrows indicate the diffusion of the substrate throughout the permeable membrane, and its conversion into the product *p*- nitrophenol (yellow arrows) that can be collected at the product outlet.

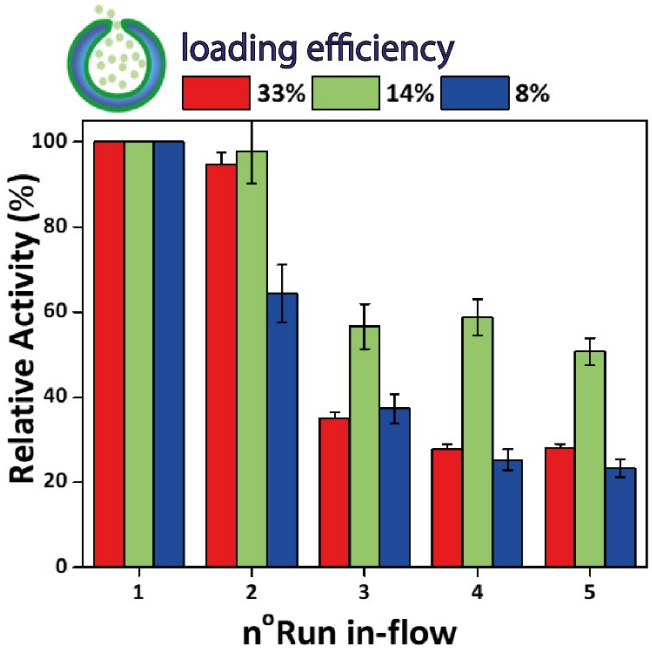

Figure S14- Relative activity of stomatocyte nanoreactors with different CalB loading during five runs in-flow (each run 13 min, 0.3 mL min^{-1}).

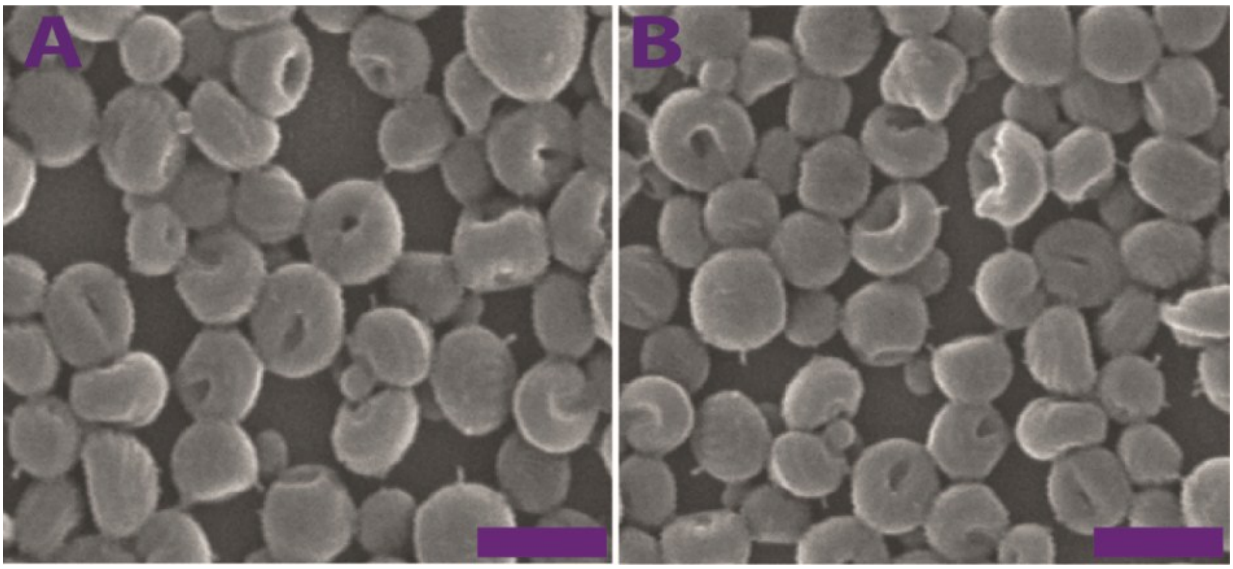

Figure S15- A) SEM images of CalB *c*-CLE*n*A after 10 runs in-flow. Scale bar corresponds to 500nm. **B)** SEM images of CalB *c*-CLE*n*A after 10 run in-flow . Scale bars correspond to 500nm.

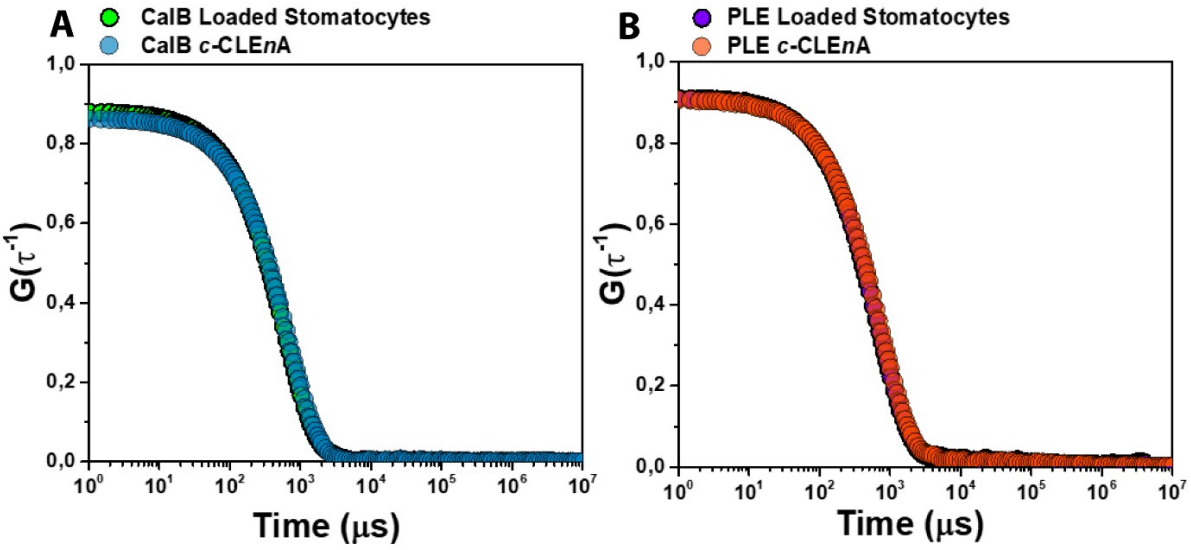

Figure S16- Correlation functions of A) CalB loaded stomatocytes (green) and CalB *c*-CLE*n*A (blue); B) correlation function PLE loaded stomatocytes (purple) and PLE *c*-CLE*n*A (orange). No significant difference was observed after *c*-CLE*n*A formation.

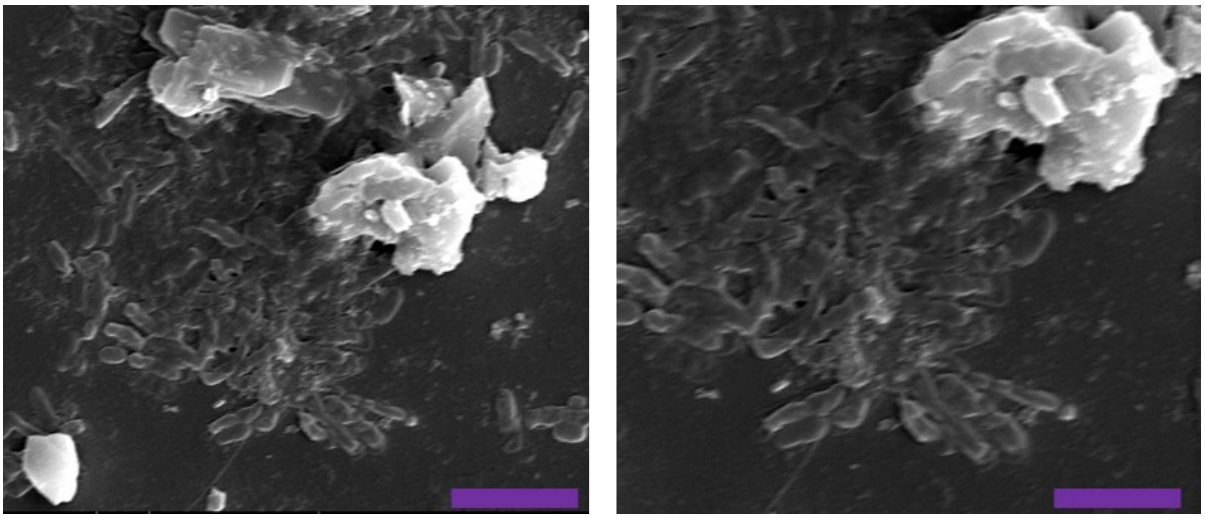

Figure S17- SEM images of non-templated CLEA formed from free PLE in solution. Please note the broad size distribution as a result of uncontrolled cross-linking. Scale bars correspond to 5 μm.

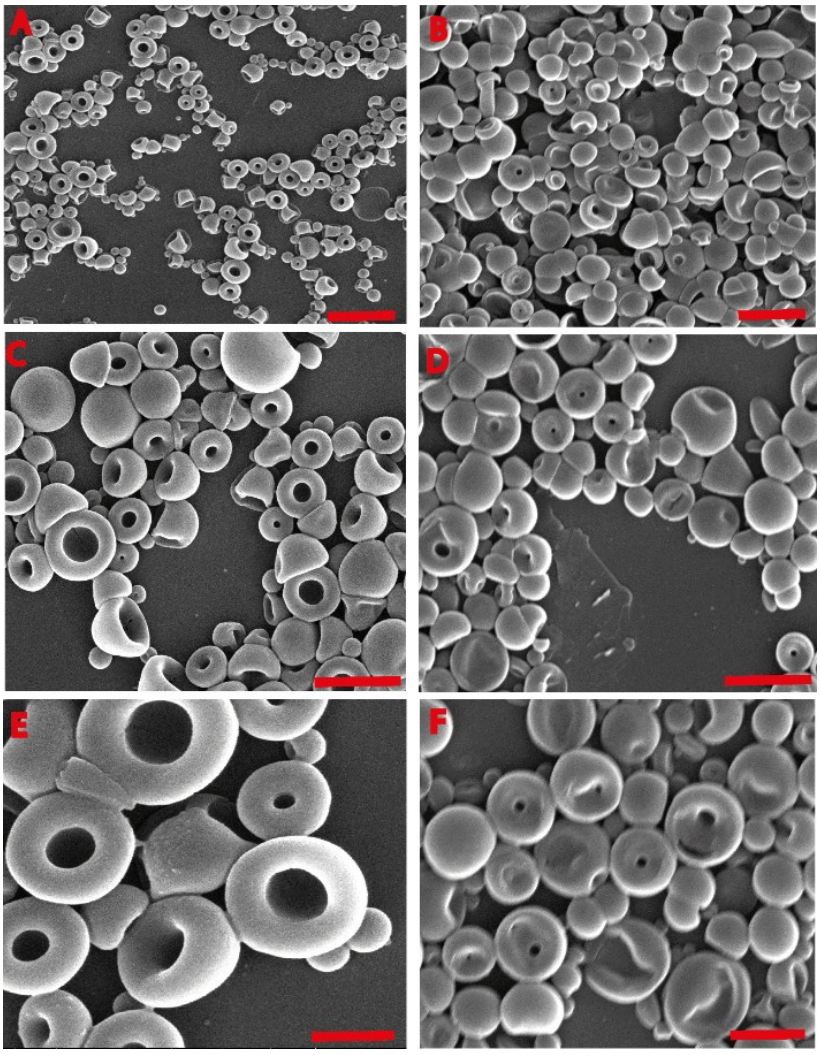

Figure S18- SEM images of A) B) C) open neck stomatocytes (used for PLE encapsulation); D) E) F) PLE *c*-CLE*n*A prepared with 50mM of genipin. Scale bar corresponds to 3 μm in A and B, 1 μm in C and D and 400nm in E and F.

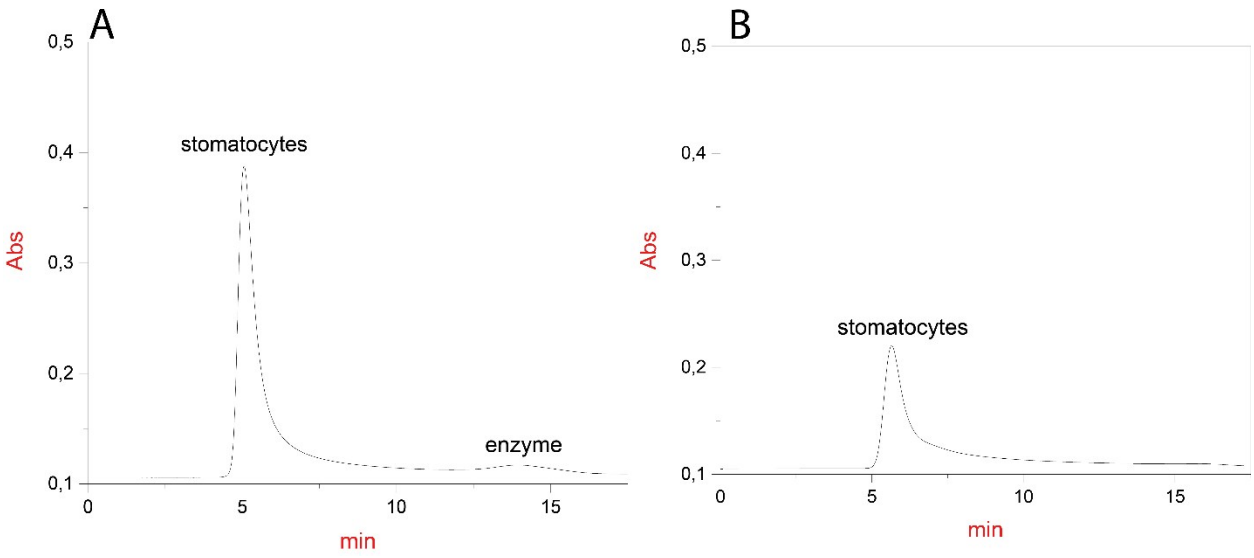

Figure S19- Example of SEC chromatograms showing A: stomatocytes (~5min) and residual enzyme after encapsulation (~13 min), B: Sample after purification showing no trace of enzyme left in solution.

References

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3 S. Khanahmadi, F. Yusof, A. Amid, S. S. Mahmod and M. K. Mahat, *J. Biotechnol.*, 2015, **202**, 153–161.

4 Y. Liu, H. Zhou, L. Wang and S. Wang, *J. Chem. Technol. Biotechnol.*, 2016, **91**, 1359–1367.