Supporting Information

Confinement of *Candida Antarctica Lipase B* in a Multifunctional Cyclodextrin-Derived Silicified Hydrogel and its Application as Enzymatic Nanoreactor

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Experimental

Materials. Lipase B from *Candida Antarctica* (CALB), recombinant from *Aspergillus oryzae* was purchased from Sigma Aldrich. CALB consists of 317 amino acid residues giving a molar mass of 33 kDa.[1] The active site of CALB is composed of a catalytic triad consisting of

nucleophilic serine, histidine and aspartate or glutamate.[2] Pluronic F127 [$PEO_{100}PPO_{70}PEO_{100}$ where PEO stands for poly(ethylene oxide) and PPO for poly(propylene oxide), average Mw 12500 g/mol], poly(4-vinylpyridine) (P4VP, average Mw 60000 g/mol), tetramethyl orthosilicate (TMOS, Mw 152.22 g/mol), chlorotrimethylsilane (CTMS, >98%, Mw 108.64 g/mol), (3-aminopropyl)-trimethoxysilane (APTMS, 97%, Mw 221.37 g/mol), glutaraldehyde (GAH, 50% in H_2O, Mw 100.11 g/mol), fluorescein *iso*-thiocyanate (FITC), 2,5-diformylfuran (DFF 97%, Mw 124.1 g/mol), 2,5-furandicarboxylic acid (FDCA 97%, Mw 156.1 g/mol), 5-formylfuran-2-carboxylic acid (FFCA, Mw 140.1 g/mol), phosphate buffer solution (50 mM, pH 7.5), ethylacetate (EtOAc) and *tert*-butanol (t-BuOH) were purchased from Sigma Aldrich. Acetate buffer solution (pH 4.6) was procured from Honeywell Fluka. Native α-cyclodextrin (α-CD, 99%, Mw 972.85 g/mol) was procured from Wacker Chemie GmbH. All chemical were used as received, without further purification.

Preparation of the silicified hydrogel. The silicified hydrogel (denoted Sihgel) was prepared according to a previously reported method with some modifications.[3] Briefly, 2.0 g of α-CD (100 mg/mL) was introduced into 20 mL of a micellar F127 solution (16 mg/mL of pluronic F127 in water). The mixture was stirred at room temperature for 15 minutes, then stored in a closed vial at 4 °C for 24 hours until a water-swollen gel formed. The pH of the hydrogel was measured to be 6.3. Subsequently, 1.42 g of TMOS (α-CD/TMOS molar ratio = 0.22) was added dropwise to the hydrogel and maintained under stirring (800 rpm) at room temperature for 2 hours. The mixture was then loaded into a 40-mL Teflon-lined autoclave and heated at 60 °C for 48 hours to complete the condensation reaction between silanols. The excess of hydrogel was removed by washing several times with water and ethanol. In some experiments, a hydrogel-free sol-gel silica (denoted Sisg), a pluronic F127-templated silica (denoted SiF127) and a α-CD-templated silica (denoted Siα-CD) were also prepared as controls.

Functionalization of the silicified hydrogel. The grafting reaction was carried out according to two methods reported earlier by Sorensen *et al.*[4] and Kao *et al.*[5] with some modifications. In a typical experiment, prior to functionalization, 250 mg of the silicified hydrogel (Sihgel) was dried under vacuum conditions at 180 °C overnight in order to remove any traces of solvent adsorbed into the pores. Then, the solid was suspended in 35 mL of anhydrous toluene, followed by successive addition of 175 µL CTMS and 122 µL APTMS. After refluxing at 100 °C for 24 hours, the solid denoted Sihgel@CA was collected by centrifugation, washed several times with toluene, then dried under vacuum and finally stored under inert (N_2) atmosphere. Reaction was performed in toluene because non-polar solvents have been reported to facilitate aggregation of silane ligands on the silica surface, thus favoring interaction with silanol groups.[6,7] Subsequently, 50 mg of the functionalized Sihgel@CA material was dispersed in 1.5 mL acetate buffer (pH 4.6) and 77 µL of glutaraldehyde (GAH) was added. After stirring at room temperature for 10 hours, the GAH cross-linked material (denoted Sihgel@CAG) was collected by centrifugation, then washed with water and ethanol and finally dried under vacuum conditions at 25 °C for 10 hours. In some experiments, for comparison purposes, the Sihgel matrix was silanized only with CTMS (Sihgel@C) or only with APTMS followed by activation with GAH (Sihgel@AG). The CTMS/SiO_2 molar ratio used in all preparations was 0.33 unless stated otherwise.

Immobilization of the enzyme. The immobilization of lipase CALB was carried out either by ionic binding or by covalent bond. In our ionic binding approach, 2 mg of lipase CALB was first dissolved in 1.5 mL acetate buffer solution (pH 4.6), then 20 mg of Sihgel@C was added. The pH of immobilization was between the isoelectric point of CALB (pI 6) and the point of zero charge of silica (PZC 2.0-3.5), so that attractive electrostatic interactions can occur between the support and the enzyme. After stirring in an ice bath for 16 hours, the supported biocatalyst was collected by centrifugation, washed with water and ethanol, then dried under

vacuum. For the immobilization by covalent bond, 20 mg of Sihgel@CAG was first dispersed in 1.2 mL acetate buffer (pH 4.6), then put in an ultrasonic bath for 2 minutes until the particles were completely disaggregated. To this solution, 2 mg of lipase CALB solubilized in 0.3 mL acetate buffer solution (pH 4.6) was added. It is very important to control carefully the pH of this reaction. In fact, the rate at which the imine bond is formed is greatest near a pH of 5.0.[8] After stirring in an ice bath for 16 hours, the supported biocatalyst was collected by centrifugation, washed several times with water and ethanol, then dried under vacuum. The same immobilization procedure was applied for the immobilization of the lipase on Sihgel@AG, SiF127@CAG, Siα-CD@CAG and Sisg@CAG supports. For all supported biocatalysts, the amount of lipase used was 2 mg for 20 mg of solid (1.33 mg/mL CALB in the immobilization solution) unless specified otherwise.

Characterization Methods. *Dynamic light scattering (DLS)* measurements were performed at 25 °C using a commercially available Malvern Instrument Zeta Nanosizer equipped with a 4 mW He-Ne red laser (633 nm). The detection angle was 173° with respect to the incident beam (backscattering). Three measurements were performed on each sample with an average of ten runs per measurement. F127/α-CD mixtures were prepared by adding various amounts of α-CD (in the concentration range of 5-100 mg/mL) to a micellar solution containing 16 mg/mL pluronic F127 (α-CD/EO molar ratios between 0.02 and 0.4). Samples were vortexed for 5 min, and then allowed to equilibrate at 4 °C for different duration times, *i.e.* 30 minutes, 4 hours or 24 hours. A 1 cm path-length quartz cell was used for the analysis. Error values determined from the standard deviations of three or six measurements were less than 20%. *Attenuated Total Reflexion Fourier Transform Infrared (ATR-FTIR)* measurements were carried out on a Shimadzu IR Prestidge-21 spectrometer equipped with a MIRacle Diamond prism. Spectra were recorded in the 4000-400 cm^{-1} region with a spectral resolution of 2 cm^{-1}. *Thermogravimetric (TG)* analyses were carried out in a Setaram TG-DTA 92 microbalance

equipped with platinum crucibles. All analyses were performed in duplicate. Measurements were performed under air (gas flow 20 mL/min) using a heating ramp of 8 °C/min, from 40 to 800 °C. For the silanized materials, the surface coverage (S_{cov}) (chain/nm^2) was calculated based on the following equation as described by Chevigny *et al.*[9]

$$S_{cov} = \left(\frac{S_{BET}}{M_{gr}\ x\ N_A}\right) x \left(\frac{W_{tot} - W_{ref}}{100 - (W_{tot} - W_{ref})}\right) \quad (1)$$

where S_{BET} is the surface area of the silicified hydrogel (nm^2/g), N_A is the Avogadro's constant (6.02 x 10^{23} /mol), M_{gr} is the molar weight of the grafted silane groups, W_{tot} and W_{ref} are the weight loss of the grafted silica and the weight loss of silica before grafting respectively.

Nitrogen adsorption-desorption isotherms were collected at -196 °C using an adsorption analyser Micromeritics Tristar 3020. Prior to analysis, 40-60 mg samples were outgassed at 180 °C overnight to remove species adsorbed on the surface. From N_2 adsorption isotherms, specific surface area (S_{BET}) was determined by the Brunauer-Emmet-Teller (BET) method. Pore size distribution and pore volume were calculated using the BJH method assuming a cylindrical pore structure. Then, from the BET surface area, the maximum grafting density (D_g^{max}) (mg/g) was determined using the following equation, assuming that a monolayer of silane was formed on the surface of the silanized hydrogel.[7]

$$D_g^{max} = \left(\frac{S_{BET}}{S_{Si}}\right)\left(\frac{M_W}{N_A}\right) \quad (2)$$

where S_{BET} is the specific surface area of the silicified hydrogel (nm^2/g), S_{Si} is the occupied surface area of a silane ligand (nm^2), M_W is the molecular weight of the organosilane and N_A is the Avogadro's constant. *Transmission electron microscopy (TEM)* observations were performed with a TECNAI microscope operating at 200 kV at medium magnification. The silica powder was deposited directly on a carbon coated copper grid. *Scanning electron microscopy (SEM)* observations were performed at 3 keV with a FEG Hitachi S-4700 microscope. Before

observation, samples were metallized with a thin layer of gold. *Confocal laser scanning microscopy (CLSM)* analysis was performed on a Zeiss LSM780 confocal microscope to determine the fluorescence signal from the fluorescein *iso*-thiocyanate (FITC)-tagged CALB after immobilization on the Sihgel@CAG support. The labelling of CALB with FITC was performed as follows: 0.5 mg of CALB solubilized in 1 mL carbonate buffer (0.1 M, pH 9) was mixed with 2 mg of FITC solubilized in 2 mL of DMSO and the mixture was incubated at 300 rpm in the dark for 16 hours. The FITC-tagged CALB was then immobilized in the silicified hydrogel modified with hydrophobic and grafting functions (Sihgel@CAG). In a control experiment, FITC was immobilized without CALB in the same functionalised material. *UV-Visible measurements* were performed using a Perkin Elmer (Lambda 19) spectrophotometer. The Bradford method was used to determine the enzyme loading in the silicified hydrogel by measuring the initial and final concentration of protein within the immobilization solution.[10] First, the calibration curves were established by measuring the ratio between the absorbance at 595 nm corresponding to the anionic blue form of the Coomassie Brilliant Blue G-250 binding to the protein, and the absorbance at 450 nm corresponding to the cationic red form of the dye. Then, the immobilization efficiency (%) and the loading capacity (wt. %) were determined by mass balance using the following equations[11]

$$\text{Immobilization efficiency} = \frac{(m - C_1V_1)}{m} \times 100 \qquad (3)$$

$$\text{loading capacity} = \frac{(m - C_1V_1)}{Ws} \times 100 \qquad (4)$$

where m (mg) is the mass of the enzyme initially added in the immobilization solution, C_1 (mg/mL) and V_1 (mL) are the enzyme concentration in the supernatant and its volume respectively and Ws (g) is the weight of the support material.

Activity measurements. The hydrolytic activity of free enzyme was 9 U/mg. 1 lipase unit corresponds to the amount of enzyme which releases 1 μmol butyric acid per minute at pH 8.0 and 40 °C using tributyrin as substrate. The catalytic activity of the immobilized enzyme was evaluated in the oxidation of DFF to FDCA as reported by Qin e*t al.*[12], with some modifications. In a typical experiment, DFF (2.48 mg, 10 mM) was dissolved in 2 mL of a EtOAc/t-BuOH (1:1 v/v) solution, then 2.0 equivalents of aqueous H_2O_2 (4.5 μL, 30% v/v) were added. The reaction was started after addition of 20 mg of supported biocatalyst. The mixture was maintained under stirring in a thermostatic bath at 40 °C. Aliquots of 2.0 equivalents H_2O_2 were added every hour up to 6 hours of reaction time. DFF and its oxidation products (FDCA and FFCA) were analysed using ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy together with high liquid performance chromatography (HPLC). ^{1}H NMR spectra were recorded on a BRUKER DPX300 Avance spectrometer of 300 MHz at 25 °C (16 scans per measurement). Products were dissolved in 600 μL DMSO prior to analysis. Typical profiles of the ^{1}H NMR spectra of isolated products and reaction mixture are shown in Figures S8-S10. DFF conversion (%), FFCA yield (%) and FDCA yield (%) were calculated according to the following equations:

$$DFF\ Conversion = 100 - \left(\frac{A_{DFF}/2}{A_{FDCA}/2 + A_{FFCA} + A_{DFF}/2} \times 100\right) \quad (5)$$

$$FFCA\ yield = \frac{A_{FFCA}}{A_{FDCA}/2 + A_{FFCA} + A_{DFF}/2} \times 100 \quad (6)$$

$$FDCA\ yield = \frac{A_{FDCA}/2}{A_{FDCA}/2 + A_{FFCA} + A_{DFF}/2} \times 100 \quad (7)$$

where A_{FDCA}, A_{FFCA} and A_{DFF} represent the integrated areas of peak a: δ = 7.30 ppm (s, 2H, Ar) from FDCA, peak b: δ = 7.60 ppm (d, 1H, Ar) from FFCA and peak a: δ = 7.67 ppm (s, 2H, Ar) from DFF, respectively. The factor 2 derives from the fact that FDCA and DFF have two

protons on their furan ring, whereas there is only one proton on the furan ring of FFCA. *HPLC analyses* were performed on a Perkin Elmer Flexar apparatus. The column used was an Aminex HPX-87H (300 mm length x 7.8 mm diameter) heated at 60 °C. Acetic acid (0.2 %) was used as the mobile phase at a flow rate of 0.6 mL/min. Aliquots of 1 μL of the sample were injected and analysed at a wavelength of 284 nm using a photodiode array detector. The amounts of DFF, FFCA and FDCA were determined using standard calibration curves. Typical calibration curves and chromatograms of the isolated products and the reaction mixture are shown in Figures S11-S13.

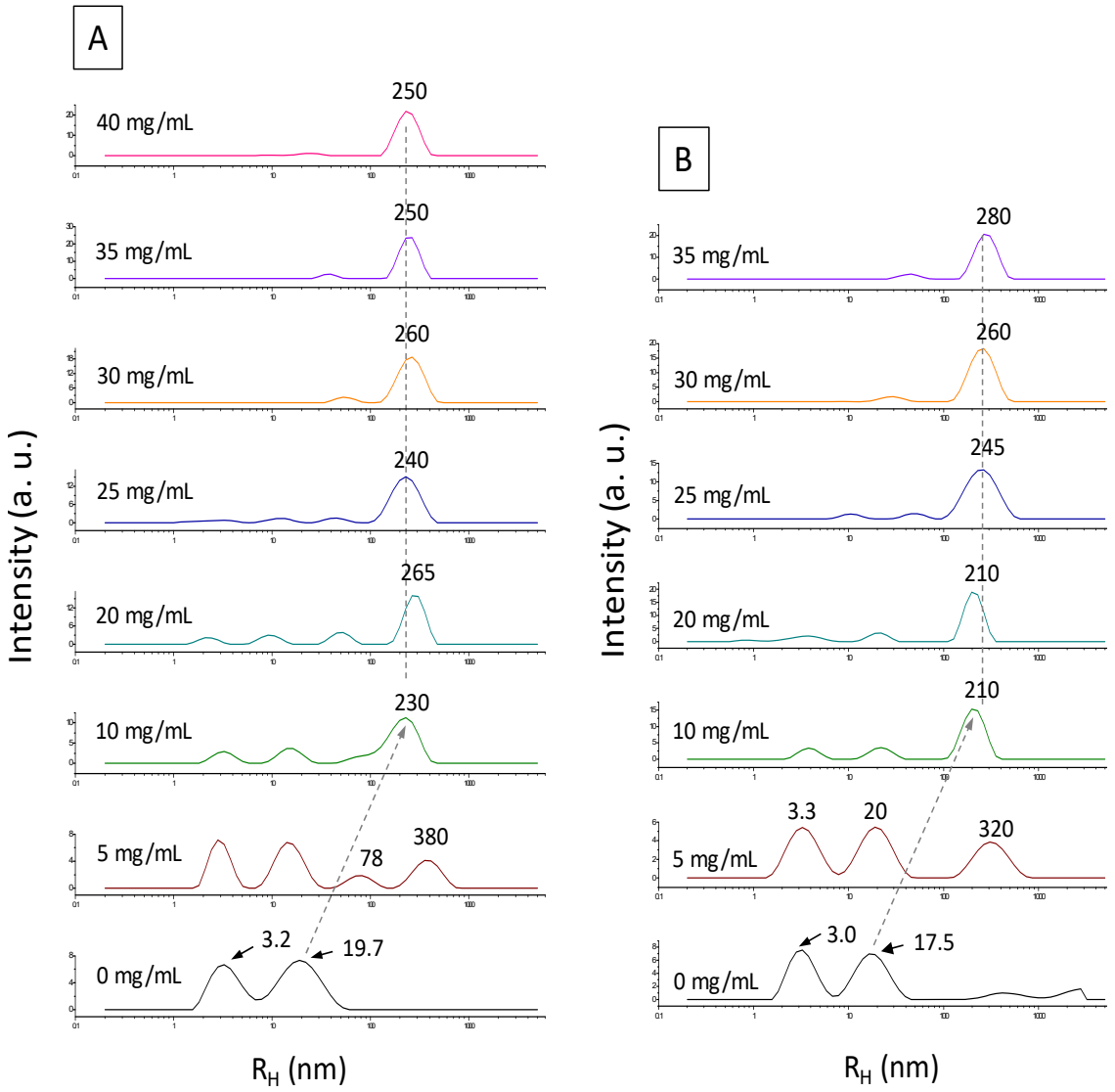

Figure S1. Apparent hydrodynamic radius distributions of the scattered intensity at 25 °C for the supramolecular assemblies prepared with 16 mg/mL pluronic F127 and increasing concentrations of α-CD. (A) from 0 to 40 mg/mL after 4 hours at 4 °C; (B) from 0 to 35 mg/mL α-CD after 24 hours at 4 °C.

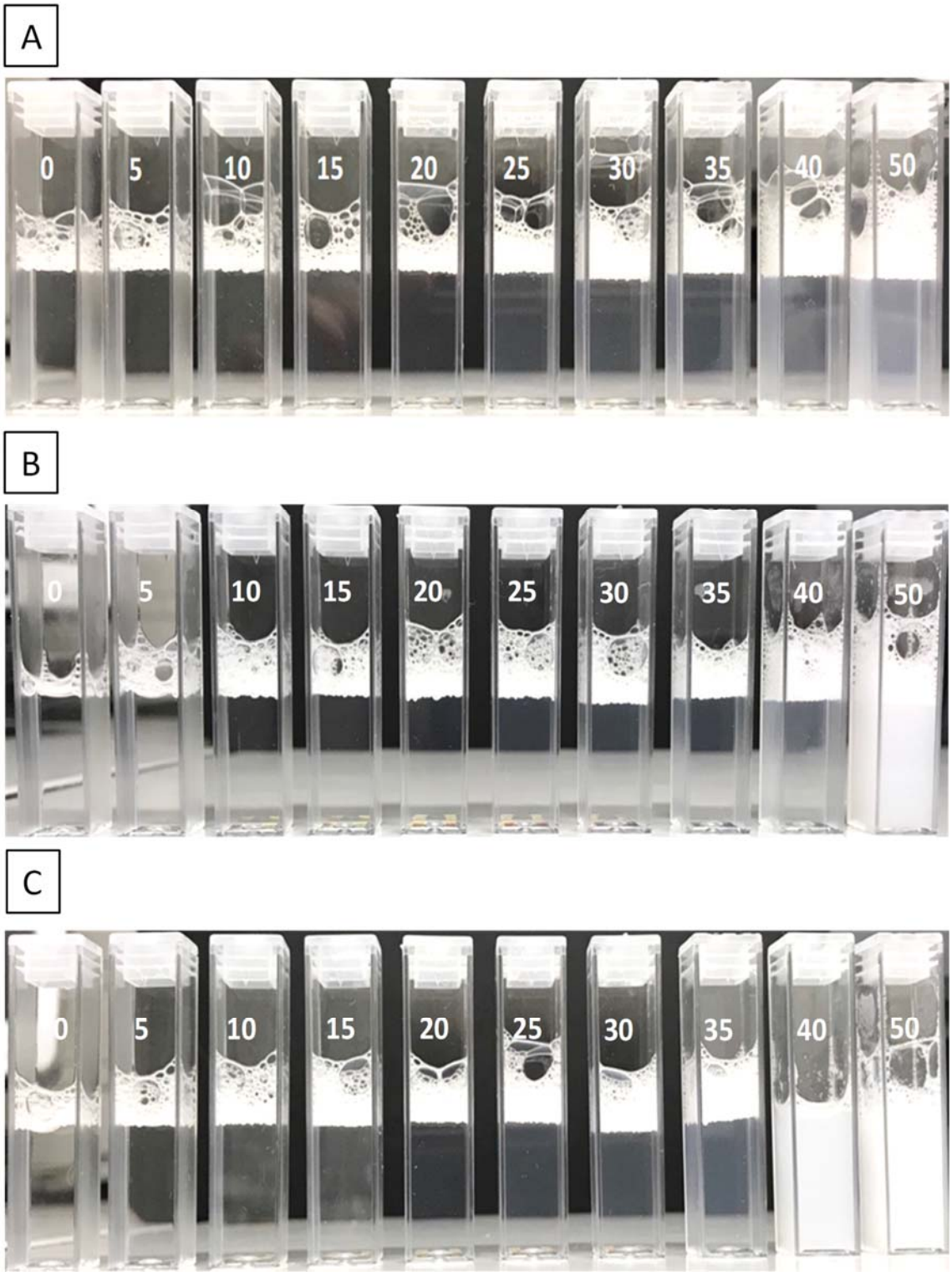

Figure S2. Visual aspect of the supramolecular assemblies prepared with 16 mg/mL pluronic F127 and increasing concentrations of α-CD (from 0 to 50 mg/mL) after (A) 30 minutes at 4 °C; (B) 4 hours at 4 °C and (C) 24 hours at 4 °C.

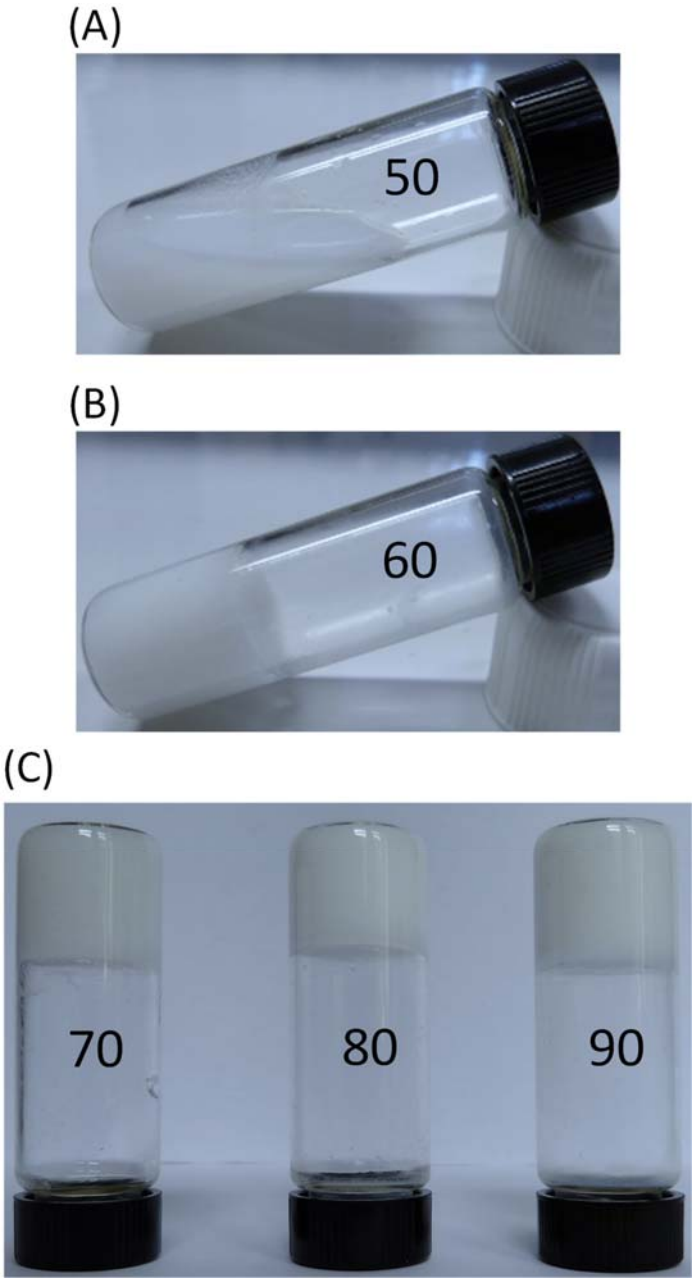

Figure S3. Visual aspect of the pluronic F127/α-CD mixtures prepared with 16 mg/mL F127 and increasing amounts of α-CD after 72 h at 4 °C: (A) 50 mg/mL α-CD, (B) 60 mg/mL α-CD and (C) 70, 80 and 90 mg/mL α-CD.

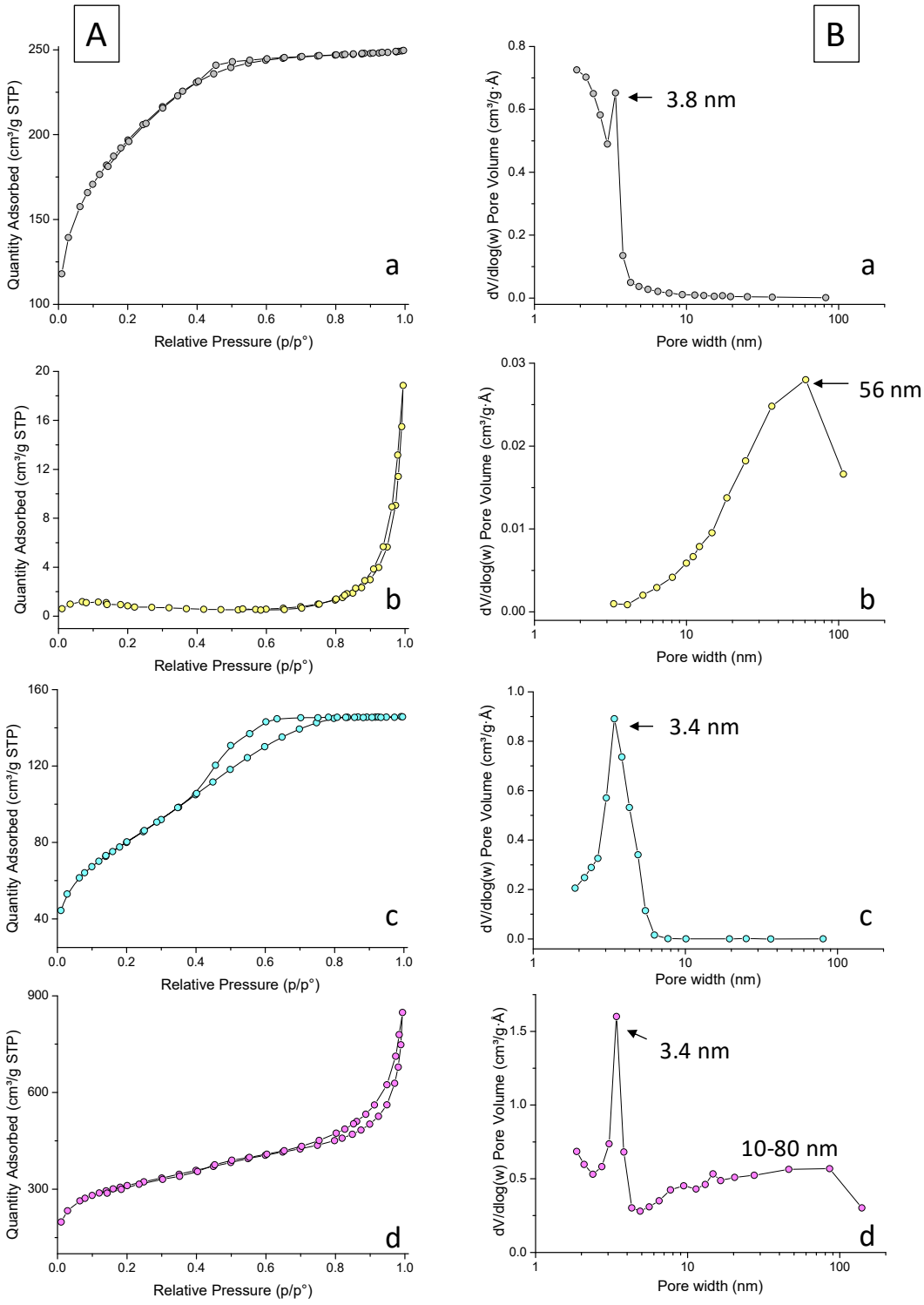

Figure S4. N_2-adsorption isotherms (A) and corresponding pore size distributions (B) of the sol-gel silica (a), pluronic F127-templated silica (b), α-CD-templated silica (c) and the silicified F127/α-CD hydrogel calcined at 500 °C (d).

Table S1. Parameters deduced from N_2-adsorption and TG analysis for the silicified hydrogel before and after modification with different functional groups and lipase immobilization.

Sample	N_2-adsorption				TG analysis	
	S_{BET}[a] ($m^2.g^{-1}$)	V_p[b] ($cm^3.g^{-1}$)	D_p[c] (nm)	% µpore[d]	*total weight loss (%)*[e]	*effective weight loss (w%)*[f]
Sisg	634	0.219	3.8 and <2.0	14.8	4.2	-
Siα-CD	287	0.198	3.4	0.1	*n/a*	*n/a*
SiF127	3	0.029	56	18.9	*n/a*	*n/a*
Sihgel-T500	1083	1.090	3.4 and 10-80	15.6	2.9	-
Sihgel	136	0.156	3.8 and 20-50 nm	7.1	17.8	13.6
Sihgel@C	82	0.136	3.8 and 20-50nm	4.2	22.3	4.5
Sihgel@A	*n/a*	*n/a*	*n/a*	*n/a*	24.4	6.6
Sihgel@AG	86	0.116	3.8 and 20-50 nm	14.7	29.5	11.7
Sihgel@CAG	54	0.139	3.8 and 20-50nm	2.6	36.2	18.4
Sihgel@CAG@CALB	39	0.094	3.8 and 20-40 nm	3.0	41.9	5.7

[a]BET specific surface area determined in the relative pressure range 0.1-0.25, [b]cumulative pore volume (BJH), [c]pore size calculated from BJH method, [d]% micropores = (V_{micro}/V_{cum})*100 where V_{micro} is the micropore volume determined from the t-plot, [e]weight loss between 180 and 700 °C, [f]weight loss corrected by the decomposition profile of hydrogel-free sol-gel silica.

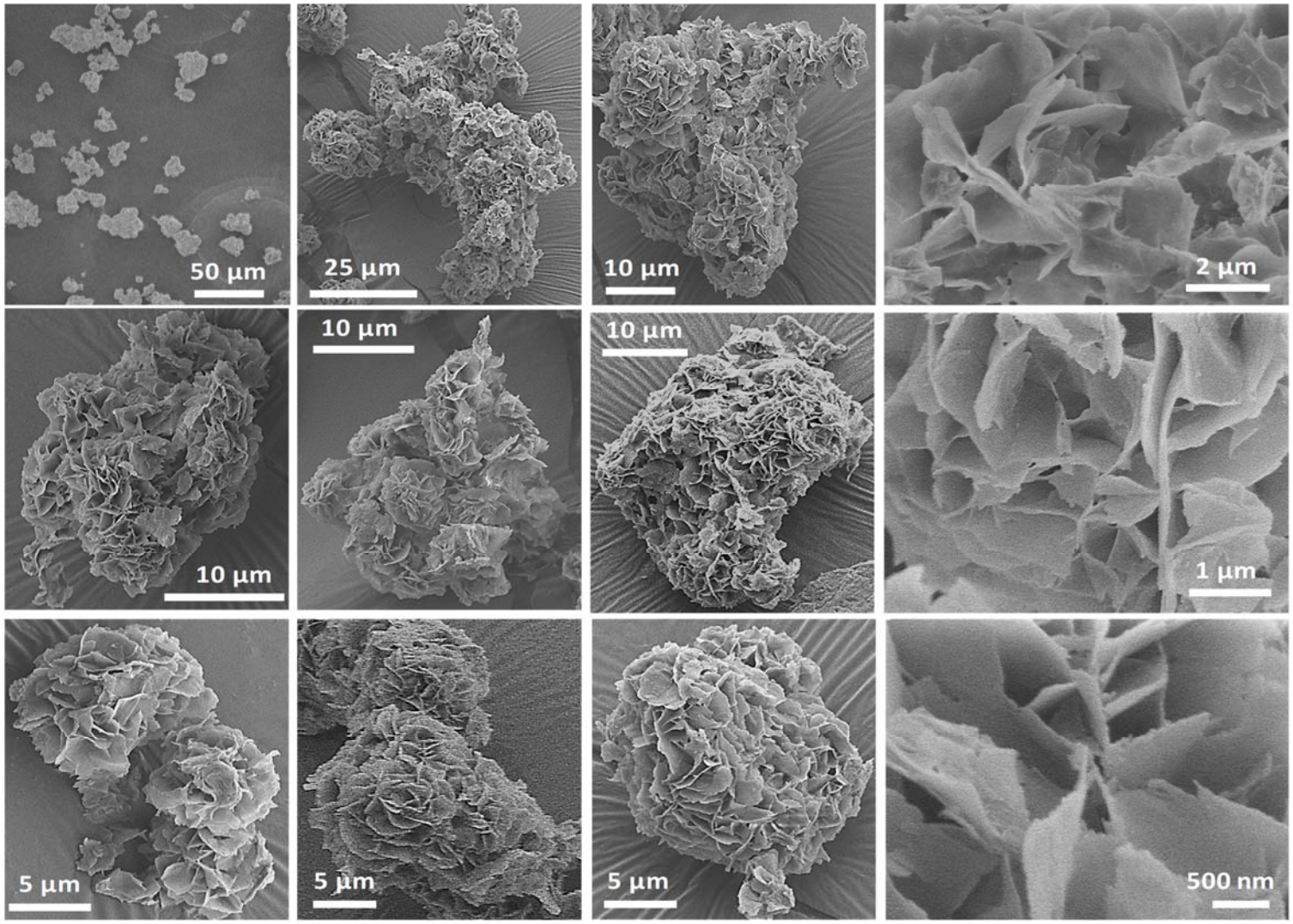

Figure S5. FE-SEM images at different magnifications of the silicified F127/α-CD hydrogel.

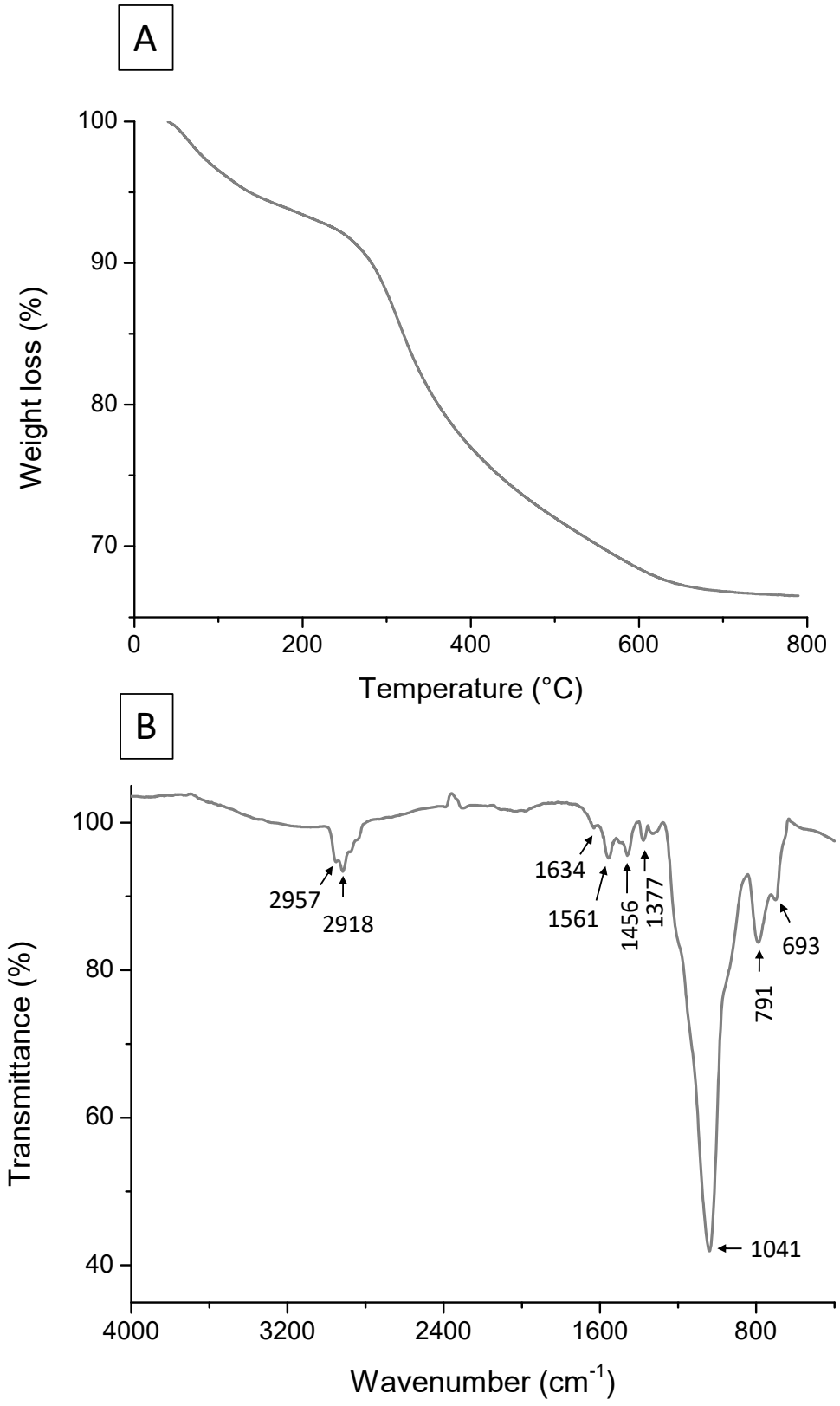

Figure S6. TG (A) and ATR-FTIR (B) curves of the APTMS-modified Sihgel (Sihgel@A).

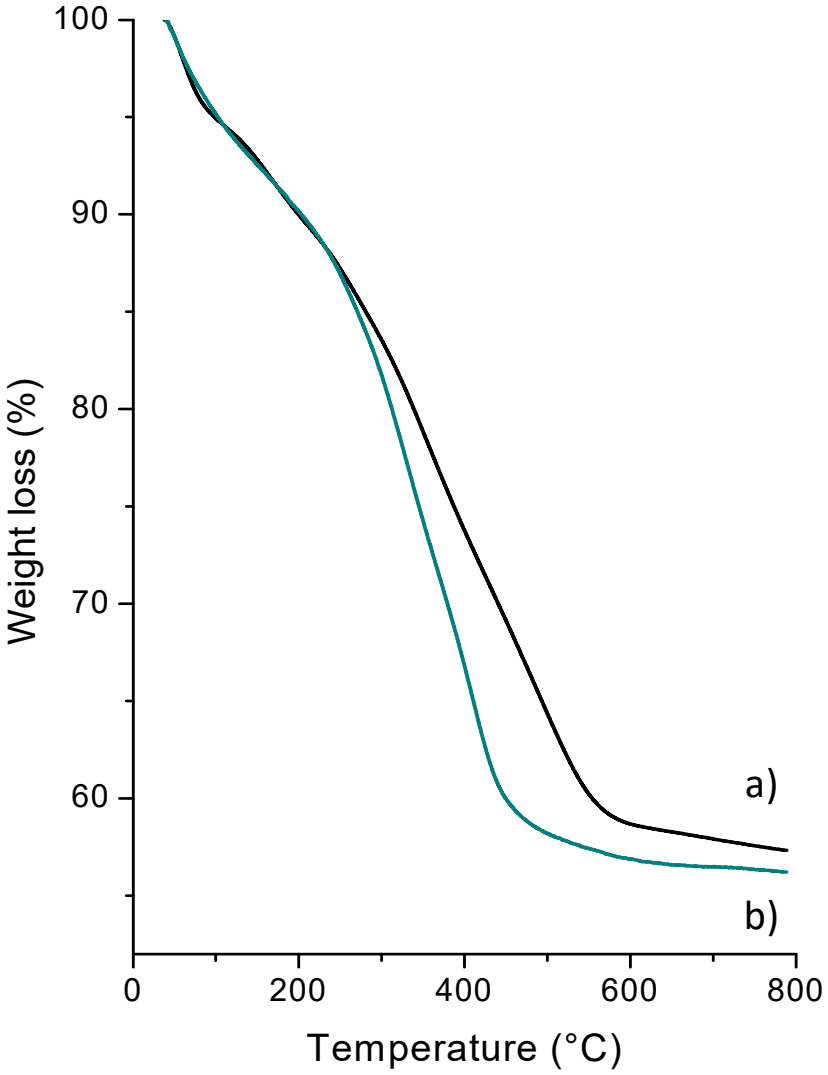

Figure S7. TG curves of Sisg@CAG material before (a) and after (b) enzyme immobilization. The loading capacity was 1.12 % CALB.

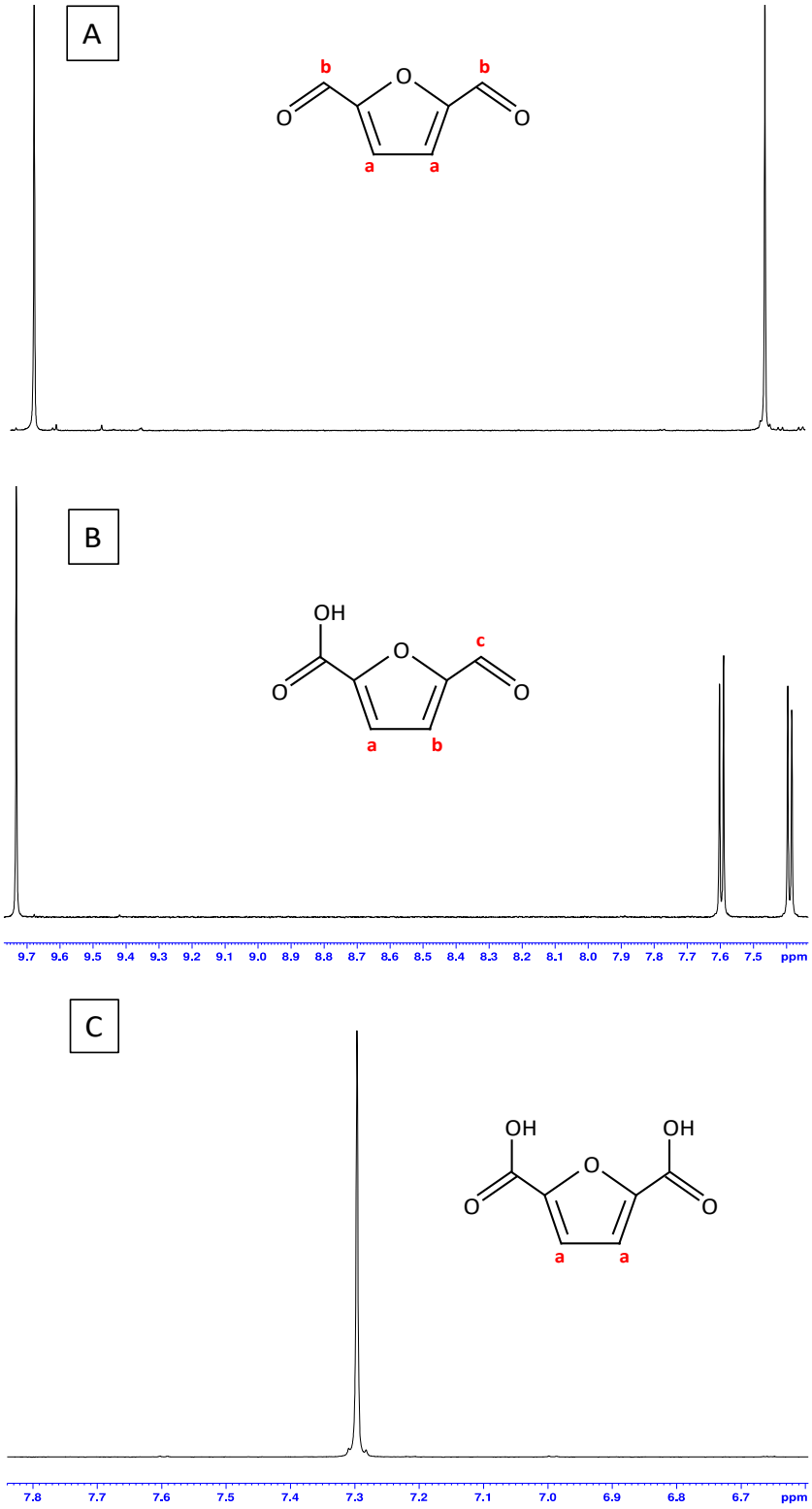

Figure S8. ^{1}H NMR (300MHz, $_{d6}$-DMSO) spectra of (A) DFF **a**: δ = 7.67 (s, 2H, Ar), **b**: δ = 9.82 (s, 2H, aldehyde); (B) FFCA **a**: δ = 7.39 (d, 1H, Ar), **b**: δ = 7.60 (d, 1H, Ar), **c**: δ = 9.73 (s, 1H, aldehyde); (C) FDCA **a**: δ = 7.30 (s, 2H, Ar).

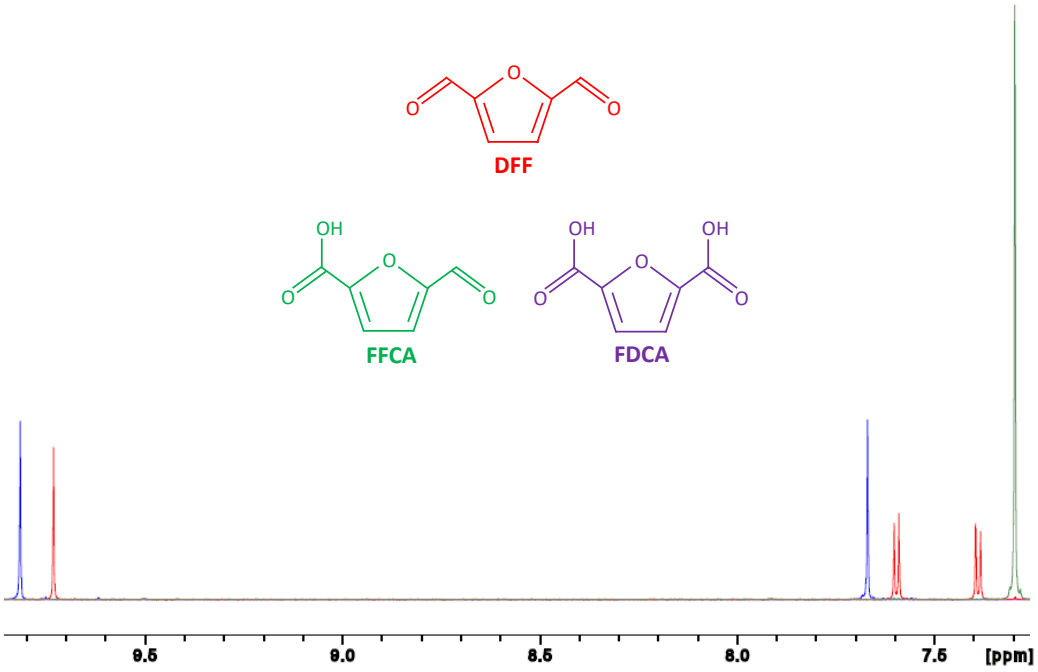

Figure S9. ^{1}H NMR spectra of DFF, FFCA and FDCA

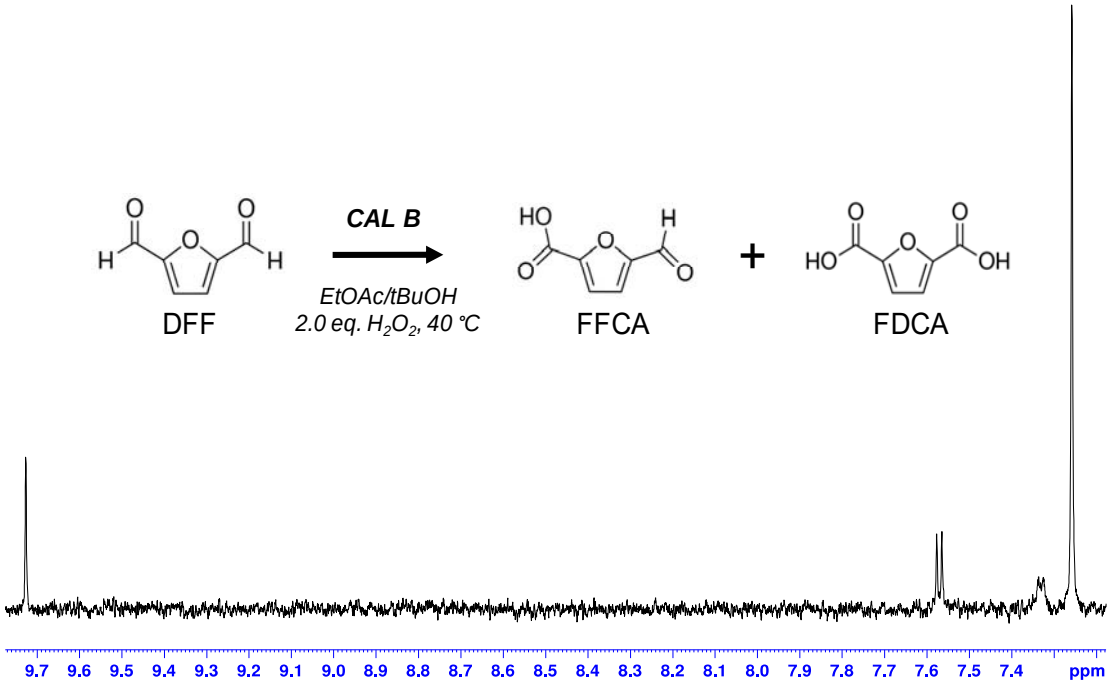

Figure S10. Typical ^{1}H NMR spectrum obtained on the oxidation of DFF: DFF conversion 100%; FFCA yield 10% and FDCA yield 90%.

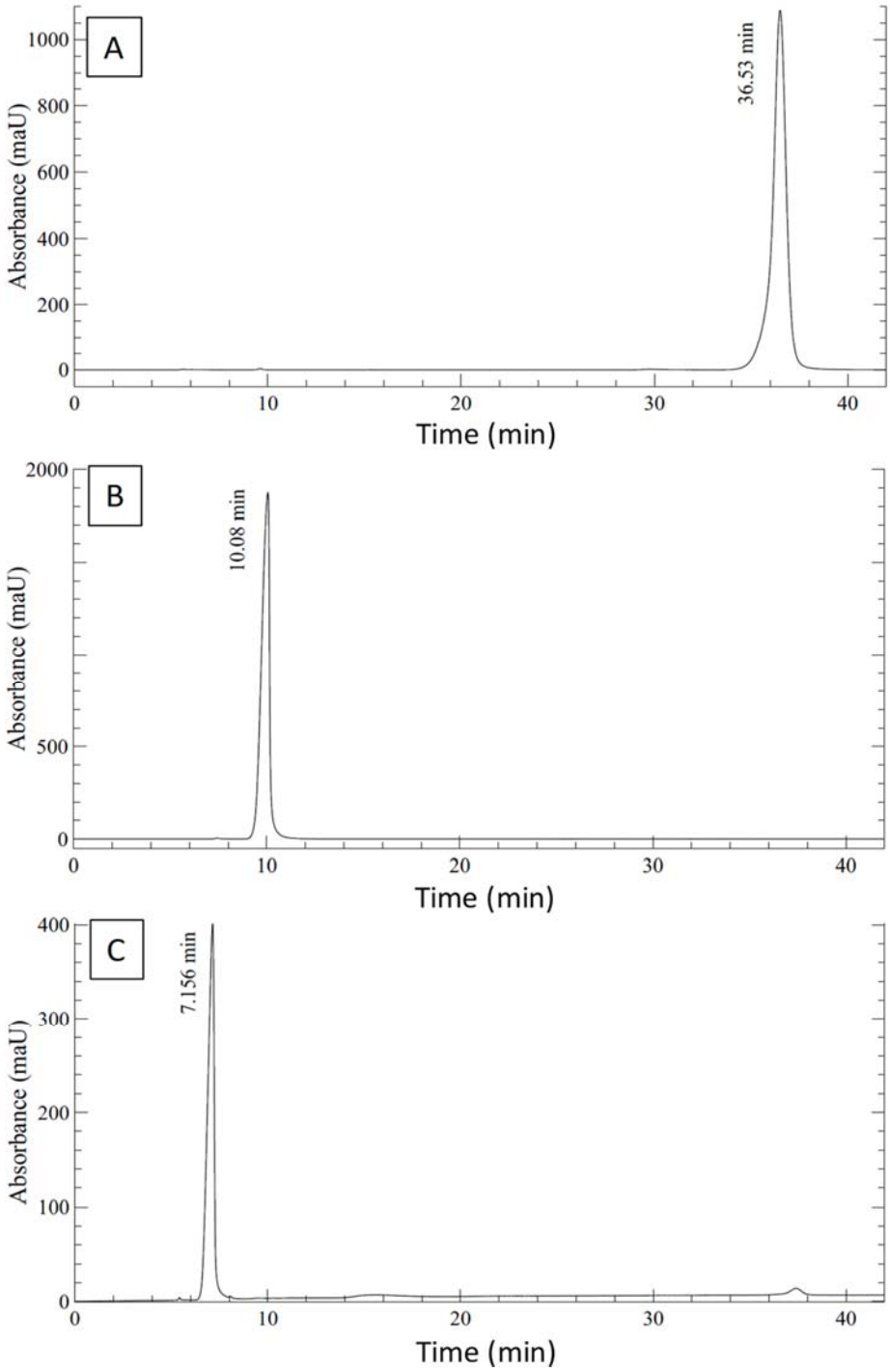

Figure S11. HPLC analysis of the isolated products: (A) DFF; (B) FFCA; (C) FDCA. The retention times of DFF, FFCA and FDCA were 36.53, 10.08 and 7.16 min, respectively. Analytic conditions: mobile phase acetic acid (0.2%), temperature 60°C, flow rate 0.6 mL/min. UV detection at 284 nm.

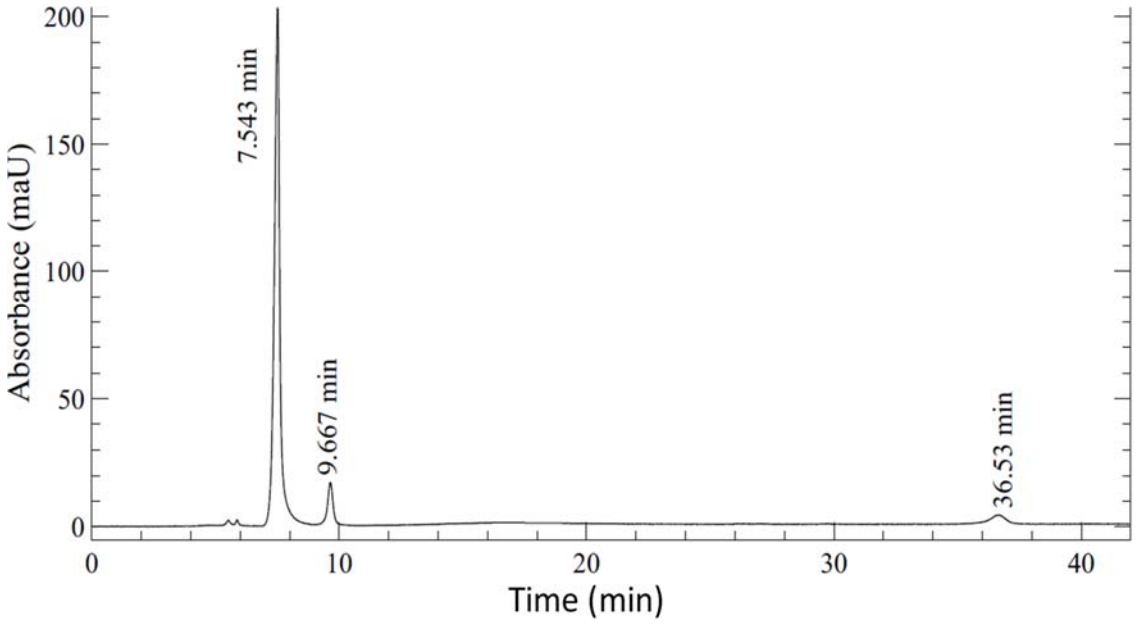

Figure S12. Typical HPLC chromatogram obtained on the oxidation of DFF to FFCA and FDCA with Sihgel@CAG@CALB. The retention times of DFF, FFCA and FDCA and the analytic conditions were the same as those described in Figure S11.

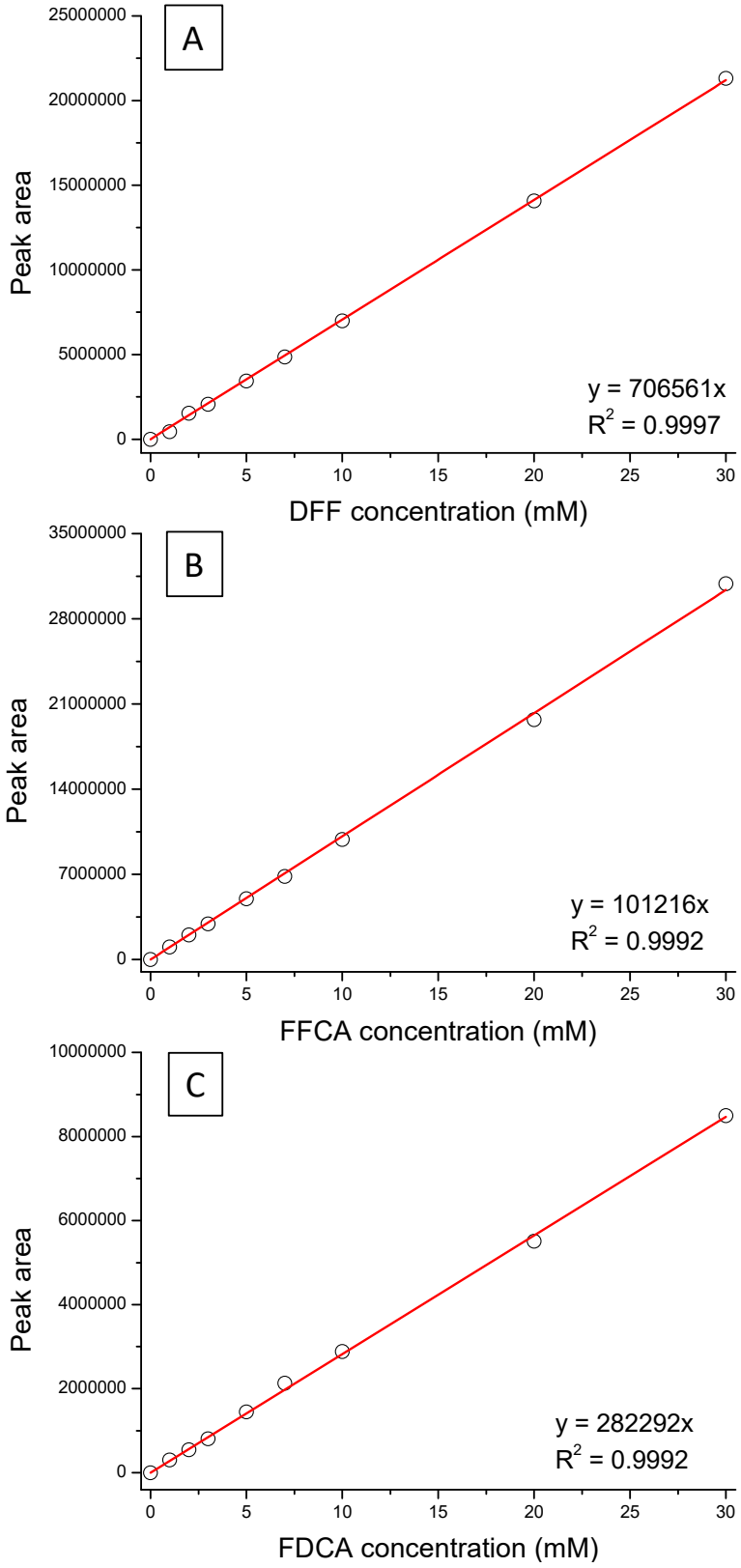

Figure S13. HPLC calibration curves for (A) DFF, (B) FFCA and (C) FDCA. Analytic conditions were the same as those described in Figure S11.

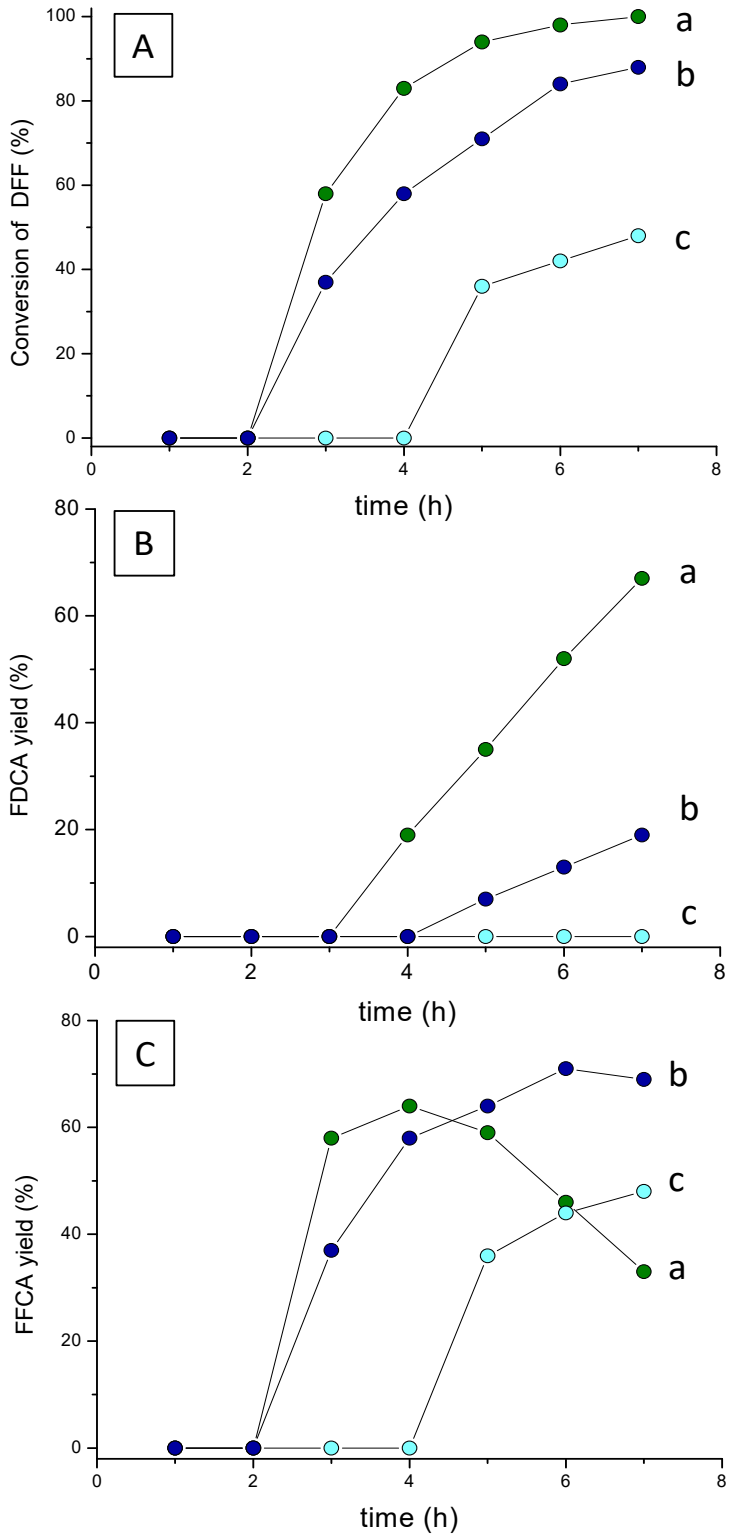

Figure S14. 7h course of the oxidation of DFF catalyzed by 2 mg lipase CALB immobilized on different supports: (a) Sihgel@CAG, (b) Sihgel@C and (c) Sihgel@AG. A) DFF conversion, B) FDCA yield and C) FFCA yield.

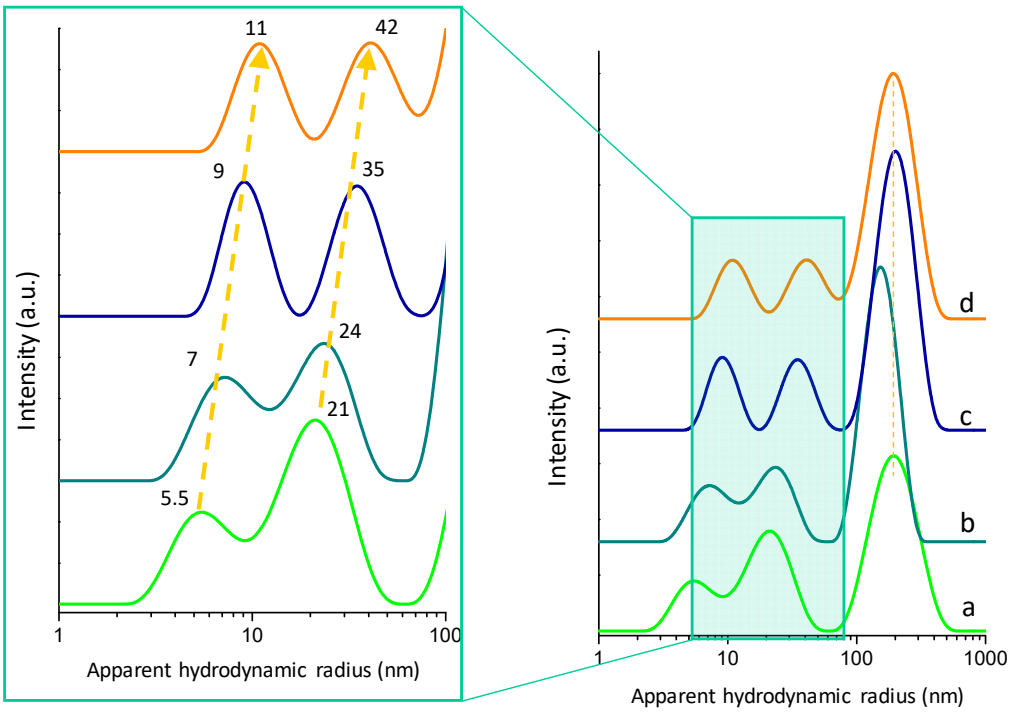

Figure S15. Apparent hydrodynamic radius distributions of the scattered intensity at 25°C for the lipase CALB solution (2 mg/mL) prepared with increasing concentrations of α-CD after 30 min at rest: a) 0 mg/mL α-CD, b) 9 mg/mL α-CD, c) 17 mg/mL α-CD and d) 23 mg/mL α-CD.

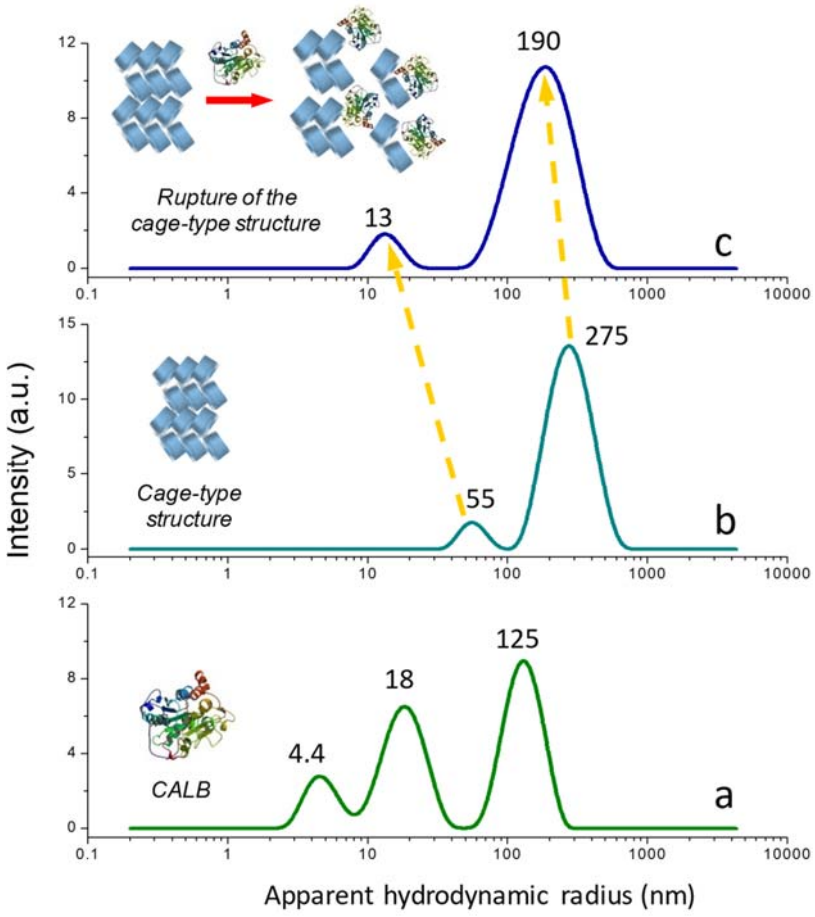

Figure S16. Apparent hydrodynamic radius distributions of the scattered intensity at 25°C for (a) the lipase CALB solution (1 mg/mL), (b) the α-CD solution (47 mg/mL) and (c) the CALB/α-CD mixture (1 mg/mL CALB, 47 mg/mL α-CD).

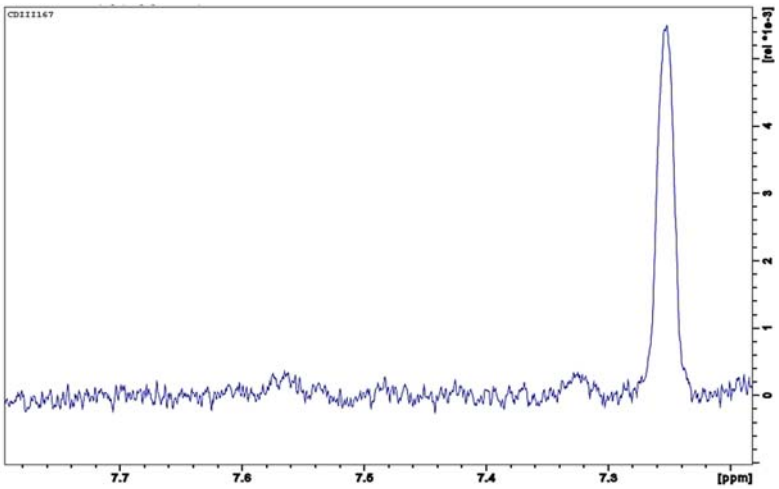

Figure S17. NMR spectrum obtained on the oxidation of DFF with the supernatant recovered after the first run using Sihgel@CAG@CALB biocatalyst. Reaction conditions: 1 mM DFF, 2 mL EtOAc/tBuOH (1:1, v/v), 0.2 equivalents aqueous H_2O_2 (30% v/v) at the beginning of the test, then after every hour for six hours, temperature 40 °C, reaction time 24 hours.

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