Supporting Information

Selective Functionalization of Microgels with Enzymes by Sortagging

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Experimental Section

Materials

The peptides H_2N-GGGRPWFWGMH-COOH and H_2N-CRPWFWGMHLPETGGRR-COOH (purity ≥ 90 %) were purchased from BIOTREND (Köln, Germany). Enzymes for gene cloning were all purchased from New England Biolabs (Frankfurt, Germany) or Fermentas (St. Leon-Rot, Germany). Primers used in polymerase chain reactions (PCR) were purchased from Eurofins MWG Operon. Microtiter plates were purchased from Greiner Bio-One GmbH, (Frickenhausen, Germany). *N*-Vinylcaprolactam (VCL, Sigma-Aldrich, 98 %) and glycidyl methacrylate (GMA, Sigma-Aldrich, 97 %) were distilled before usage in chemical synthesis. VCL was recrystallized from hexane after distillation. The crosslinker *N,N'*-methylenebis(acrylamide) (BIS, 99 %, Sigma-Aldrich, Hamburg, Germany) and the initiator 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AMPA, 97 %, Sigma-Aldrich,) were used as received from the suppliers. Indigo carmine was purchased from Sigma-Aldrich (98 %).

The swelling degree and thereby the hydrodynamic radius of the PVCL/GMA microgels are highly temperature-dependent. Unless otherwise stated, the handling temperature of all kinds of PVCL/GMA microgel samples should be not higher than 25°C.

Gene construction of GGG- and LPETGG-tagged enzymes

Overlap extension PCR was used to add the GGG- and LPETG-tags to the N- and C-termini of the enzymes, respectively. All PCR solutions (25 μL) for amplification consist of 1.25 U Phusion DNA polymerase, 10 mM dNTP mix, 10 ng plasmid template and 25 uM of each primer (forward primer and reverse primer). PCR products were treated with *Dpn*I (5 U, 37 °C, overnight) to digest the remaining plasmid template, subsequently the enzyme was heat inactivated (80 °C for 20 min). The digested samples were then purified. PCR products were transformed into the corresponding competent cells.

Primers for all the PCRs are listed in **Table S1**. The pET22b(+)-*bsla* of *Bacillus subtilis* lipase A (BSLA[1]) was used as the template to generate the GGG-BSLA construct. A two-step PCR protocol was performed: 98 °C for 30 s (1 cycle); 98 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min 30 s (5 cycles); 72 °C for 10 min (1 cycle); 8 °C for holding and mixing the forward and reverse fragments; 98 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min 30 sec (25 cycles); 72 °C for 10 min (1 cycle). Primers *Fw-ggg-bsla* and *Fw-ggg-bsla* were used in the PCR. The amplified PCR product encoded GGG-BSLA was transformed into *E. coli* BL-21 (DE3) competent cells.

Primers *Fw ggg-phytase* and *Rev ggg-phytase* were employed for the amplification of GGG-Ym-phytase using the plasmid pALXtreme-5b phytase as template.[2] PCRs protocol: 98 °C for 45 s (1 cycle); 98 °C for 45 s, 55 °C for 30 s, 72 °C for 4 min 30 s (25 cycles); 72 °C for 10 min (1 cycle). The amplified PCR product was transformed into *E. coli* BL-21 competent cells. Plasmid pET-22b(+) CueO-StrepII was used to generate GGG-CueO and CueO-LPETGGGRR constructs. PCR of CueO-LPETGGGRR was performed as previously described.[3] Primers *Fw ggg-cueo* and *Rev ggg-cueo* were employed for the amplification of GGG-CueO. The same PCR protocol as

mentioned in the construction of GGG-Ym-phytase was used. The amplified PCR products were transformed into *E. coli* Shuffle T7 competent cells.

The pET-28a plasmid including the gene of cellulase A2 variant M2 (H288F, hereafter named CelA2 M2,)[4] was used as the template to generated the construct GGG-CelA2. Primers *Fw ggg-cel-a2* and *Rev ggg-ce-a2* were used. PCR protocol: 98 °C for 30 s (1 cycle); 98 °C for 30 s, 57° C for 30 s, 72 °C for 3 min 30 s (25 cycles); 72 °C for 10 min (1 cycle). The amplified plasmid encoding GGG-CelA2 was transformed into *E. coli* BL-21 (DE3) competent cells.

The pALXtreme-1a plasmid containing the gene of P450 BM3 (F87A)[5] was used as the template to generated GGG-P450 BM3 (F87A) and P450 BM3 (F87A)-LPETGGGRR constructs. *Fw ggg-his-p450* and *Rev ggg-his-p450* were used to generate GGG-P450 BM3 (F87A). A two-step PCR was performed: 98 °C for 30 s (1 cycle); 98 °C for 30 s, 58 °C for 30 s, 72 °C for 3 min 30 s (5 cycles); 72 °C for 10 min (1 cycle). The PCR reaction was cooled to 8 °C and the forward and reverse fragments were mixed. The following step was performed using the PCR protocol: 98 °C for 30 s, 58 °C for 30 s, 72 °C for 3 min 30 s (20 cycles); 72 °C for 10 min (1 cycle). Same PCR protocol was used to construct P450 BM3 (F87A)-LPETGGGRR. Expected primers *Fw p450 lpetgggrr* and *Rev p450 lpetgggrr primers* instead of *Fw ggg-his-p450* and *Rev ggg-his-p450* were used. Plasmids encoded P450 BM3 genes were transformed into *E. coli* BL-21 (DE3) Lac^{IQ} competent cells.

Production of sortase, GGG- and LPETGGGRR-tagged enzymes

The expression and purification of Sa-SrtA rM4 and CueO-LPETGGGRR were implemented as previously reported.[3] The expression of GGG-BSLA was performed as previously described.[6] Briefly, pre-cultures (5 mL LB media, 100 μg/mL ampicillin) were inoculated from a glycerol

stock and incubated (16 h, 250 rpm, 37 °C, 70% humidity). The main culture (200 mL auto-induction media, 100 µg/mL ampicillin) in 1 L flask was inoculated with 1 mL pre-culture (18 h, 250 rpm, 37° C, 70 % humidity). GGG-Ym-Phytase was expressed in TB medium. Main culture (200 mL TB medium, 50 µg/mL ampicillin) in 1 L flask was inoculated with pre-culture with an initial OD_{600} at 0.05 and incubated (250 rpm, 37 °C, 70% humidity). When OD_{600} reached 1.0, IPTG (200 µL, 1M) was added into the main culture and subsequently cultivated (16 h, 250 rpm, 30 °C, 70 % humidity). GGG-CelA2 (M2) was also expressed in TB medium. Briefly, the main culture (200 mL TB medium, 50 µg/mL kanamycin) in 1 L flask was inoculated with pre-culture with an initial OD_{600} at 0.05 and cultivated (250 rpm, 37 °C, 70% humidity) until OD_{600} reached 0.8. Cells were induced by adding IPTG (200µL, 1M) and subsequently incubated (16 h, 250 rpm, 30°C, 70% humidity). GGG-P450 BM3 (F87A) and P450 BM3-LPETGGGRR (F87A) were expressed based on a previously implemented protocol.[5] Briefly, main culture (200 mL TB medium, 50 µg/mL kanamycin) was mixed with 200 µL 1M trace elements solution in 1 L flask. Pre-culture was inoculated into the main culture with an initial OD_{600} at 0.05. Main culture was incubated (250 rpm, 3 h, 30 °C, 70 % humidity) until OD_{600} reached 0.8. Cells were induced by adding IPTG (200 µL, 100 mM), δ-aminolevulinic acid anhydride (ALA, 200 µL, 500 mM) subsequently cultivated (24 h, 250 rpm, 25 °C, 70 % humidity).

Cells were harvested by centrifugation (30 min, 3220 *g*, 4 °C, Eppendorf centrifuge 5810 R). The obtained cell pellets were frozen (- 20°C, 24 h) in 1 g aliquots and then thawed and dispersed in buffer (10 mL, pH 7.5, 50 mM, Tris/HCl). Cells were disrupted by sonication (60% amplitude, 12 cycles, 15 seconds per cycle, intervals 15 seconds, performed on ice). After centrifugation (1 h, 3220 *g*, 4 °C, Eppendorf centrifuge 5810 R) the supernatant of cell-free lysate was used for SDS-PAGE analysis (**Figure S1**).

Purification of GGG-CelA2 and GGG-P450 BM3 F87A was performed by using Ni Sepharose™ 6 Fast Flow purification kits (GE Healthcare, Freiburg, Germany). Procedures were implemented according to the manufacturer´s protocol. Elution fractions were firstly checked by SDS-PAGE. The fractions with pure enzymes were pooled and dialyzed overnight (pH 7.5, 4 °C, 50 mM, Tris/HCl buffer) by using Spectra/Por dialysis tubing (Spectrum Laboratories, Inc., CA, USA). Amicon ultra-15 centrifugal filter units (10 kDa cut-off, Merck Millipore Ltd, Tullagreen, IRL) were used for preparation of concentrated protein samples. Copper ions ($CuCl_2$, 2 mM) were added and incubated with purified CueO-LPETGGGRR for at least 24 h before using.

Activity assays of GGG- and LPETG-tagged enzyme

The activity of BSLAs was measured colormetrically using *p*-nitrophenol (absorbance detected at 410 nm).[6] In detail, *p*-nitrophenyl butyrate (pNPB, 10 μL, 10 mM in DMSO) was pipetted into a 96-well MTP (transparent, flat bottom, GreinerBio-one, Frickenhausen, Germany) and incubated with buffer (185 μL, pH 7.5, 50 mM, Tris/HCl). The reaction was initiated by adding 5 μL supernatant of BSLA cell-free lysate. Plates were stirred briefly and the absorbance was continuously recorded (410 nm, room temperature, 10 min, Tecan Infinite M1000 PRO plate reader, Tecan Group AG, Männedorf, Switzerland). Activity (slope of the absorbance in the linear ange, AU/h) of BSLA, GGG-BSLA is shown in **Figure S2a**.

Activity measurement of *Yersinia mollaretii* phytase (Ym-Phytase) was performed based on a fluorogenic 4-methylumbelliferylphosphate (4-MUP) assay.[2] Firstly, cell-free lysate (10 μL) was pipetted into 96-well MTP (black, flat bottom, GreinerBio-one). In the next step, buffer (40 μL, 100 mM, pH 5.5, sodium acetate) was added. Reaction was initiated by mixing with MUP solution (50 μL, 1 mM MUP dissolved in 100 mM, pH 5.5, sodium acetate buffer). Fluorescence was continuously recorded (λ_{exc} = 360 nm; λ_{em} = 465 nm, 15 min, room temperature, gain = 100,

Tecan infinite 1000 PRO plate reader). Data of Ym-phytase activity (slope of the fluorescence in the linear range, RFU/s) is given in **Figure S2b**.

Activity of CueO laccase cell-free lysates was monitored using a 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay as previously described.[3] In brief, ABTS (198 µL, 3 mM dissolved in sodium citrate buffer, pH 3, 100 mM) was pipetted in 96-well MTP (transparent, flat bottom, GreinerBio-one). Reaction was initiated by adding cell-free lysate (2 µL). Absorbance was recorded (420 nm, room temperature, 30min, Tecan Infinite M1000 PRO plate reader). The obtained data (data not shown) is comparable as previously reported.[3]

To measure the activity of CelA2 (M2) cell-free lysates, a 4-methylumbelliferyl-β-D-cellobioside (4-MUC) assay was employed.[4] In brief, cell-free lysate (3 µL) was incubated in buffer (47 µL, pH 7.2, 200 mM, phosphate buffer). Reaction was initiated by adding MUC solution (50 µL, 1 mM 4-MUC). Plates were stirred briefly (5 seconds) and the florescence was continuously recorded (λ_{exc} = 330 nm; λ_{em} = 450 nm, room temperature, 15 min, gain = 100, Tecan infinite 1000 PRO plate reader). Activities (slope of the fluorescence in the linear range, RFU/s) of Cel-A2 (M2) and GGG-CelA2 (M2) were calculated and data is shown in **Figure S2c**.

The compound 7-benzoxy-3-carboxycoumarin ethyl ester (BCCE) was used as the substrate to determine the activity of P450 BM3 [7]. In detail, cell-free lysate (5 µL) was firstly incubated in buffer (88 µL, pH 8.0, 50 mM, Tris/HCl) in 96-well MTP (black, flat bottom GreinerBio-one). 2 µL of BCCE (2 mM in DMSO) were subsequently pipetted into the MTP and afterwards incubated (5 min, 800 rpm, room temperature). Reaction was initiated by the addition of NADPH (5 µL, 10 mM). The fluorescence was recorded (λ_{exc}: 400 nm, λ_{em}: 440 nm room temperature, gain = 120, Tecan Infinite M1000 PRO microtiter plate reader). Activities (slope of the

fluorescence in the linear range, RFU/s) of P450-BM3 F87A, GGG-P450-BM3 F87A, and P450-BM3-LPETGGGRR F87A were calculated and data is shown in **Figure S2d**.

Synthesis of peptide-tagged PVCL/GMA microgels

UV/vis Spectroscopy. To proof the coupling of the GGG-peptide sequences to the PVCL/GMA microgels, UV/vis spectroscopy was performed with a Photometer Cary 100 Bio from Agilent. The spectra were monitored in a wavelength range between 200 nm and 800 nm at a temperature of 25 °C. Due to the microgel samples being dispersed in Tris/HCl buffer (50 mM, pH 7.5), this buffer was also used as a reference. Microgel samples were diluted so that the maximum absorbance was below 1.5 for all measurements. UV-Cuvettes micro from BRAND (light path 10 mm) was used.

Dynamic Light Scattering. The hydrodynamic radius of the microgel samples as well as the particle size distributions were investigated with dynamic light scattering (DLS) using an ALV/CGS-3 Compact Goniometer System with an ALV/LSE 5004 Tau Digital Correlator and a JDS Uniphase laser operated at 632.8 nm. The scattering angle was fixed to $\theta = 90°$. All microgel samples were diluted with ultrapure water until the dispersions were not turbid and then filtered through a 1.2 µm PET filter. Microgel sizes were measured at 20 °C and 50 °C to monitor the temperature-responsiveness.

Raman spectroscopy. Raman spectroscopy was used to proof the successful coupling of the peptide sequences to the PVCL/GMA microgels. Raman spectra were recorded with a Bruker RFS 100/S Raman spectrometer with a Nd:YAG laser (λ=1064 nm). The lyophilized microgel samples were pressed into aluminum pans. The measurements were carried out with a spectral

resolution of 4 cm^{-1}, power of 200 mW and 1000 scans. The software OPUS 4.0 was utilized for the measurements and data analysis.

Sortagging of enzymes to the surface of peptide-tagged microgels

Buffer A (5 mM $CaCl_2$, 150 mM NaCl, 50 mM, pH 7.5, Tris/HCl) is generally employed in sortagging.[3, 8] All the selected enzymes except P450 BM3 F87A are stable in buffer A. Activity of P450 BM3 was strongly inhibited by $CaCl_2$ and NaCl (**Figure S3**). Therefore, $CaCl_2$ and NaCl were not employed in P450 BM3 F87As immobilization experiments.

GGG-tagged enzymes immobilization on PVCL/GMA-LPETGGRR microgels

The protocol for immobilization of GGG-tagged enzymes on PVCL/GMA-LPETGGRR microgels was performed based on our previously established protocol.[9] Compounds and reaction conditions are summated in **Table S4**. In detail, PVCL/GMA-LPETGGRR microgels (0.1 mg) were dispersed in buffer A (240 μL). The supernatant (150 μL) of GGG-BSLA, GGG-Ym-phytase, or GGG-CelA2 (M2), was pipetted into the PVCL/GMA-LPETGGRR microgels suspension. Reaction was initiated by supplementing SrtA rM4 (10 μL, 50 μM). Regarding the immobilization of GGG-P450 F87A, PVCL/GMA-LPETGGRR microgels (0.1 mg) were dispersed in buffer (160 μL, 50 mM, pH 7.5, Tris/HCl) and mixed with 150 μL of GGG-P450 BM3 F87A cell free lysate. The immobilization was initiated by adding SrtA rM4 (50 μL, 40 μM). Two negative controls were performed in parallel for each immobilization experiment. In the "no tag" control, PVCL/GMA microgels (0.1 mg) were dispersed instead of PVCL/GMA-LPETGGRR microgels. In the "No sortase" control, buffer (50 mM, pH 7.5, Tris/HCl) was added instead of SrtA rM4. The reaction mixtures were incubated (20° C, 16 h, 800 rpm) for immobilization of GGG-BSLA, GGG-Ym-phytase or GGG-CelA2 (M2) or incubated (20°C, 24 h, 800 rpm) for immobilization of GGG-P450 BM3 F87A. After sortagging, samples were

centrifuged (15 °C, 10 min, 9390 *g*). The supernatant was pipetted out and the pellet was dispersed (400 μL, pure water) and followed by incubation in a shaker (600 rpm, 5 min, room temperature). The same procedure was repeated one more cycle. The microgel pellet was recovered again by centrifugation (15 °C, 10 min, 9390 *g*). The microgels with immobilized enzymes (GelZyms) were re-dispersed (200 μL pure water, concentration of microgels in water is 0.5 mg/mL) and used to evaluate the immobilized enzyme activity.

The pNPB assay [6] was employed to measure the activity of GGG-BSLA immobilized on PVCL/GMA-LPETGGRR (BSLA-GelZyms). In short, re-dispersed BSLA-GelZyms (10 μL in pure water) were mixed with buffer (180 μL, 50 mM, pH 7.5, Tris/HCl) in a 96-well MTP (transparent, flat bottom GreinerBio-one). Reaction was initiated by adding pNPB substrate (10 μL, 10 mM). Plates were stirred (5 seconds) and the absorbance was continuously recorded (410 nm, room temperature, 10 min, Tecan Infinite M1000 PRO plate reader). Activity (slope of the absorbance in the linear ange, AU/h) was calculated and data is shown in **Table 1**. Five washing cycles were carried out with BSLA-GelZyms to minimize the unspecific binding of proteins within microgels. The activity of the immobilized BSLA was measured after each washing cycle. Data was showed in **Figure S4**.

MUP assay[2] was employed to measure the activity of immobilized GGG-Ym-phytase (Ym-phytase-GelZyms). Re-dispersed Ym-phytase-GelZyms (50 μL in pure water) were pipetted into 96-well MTP (black, flat bottom, GreinerBio-one). Reaction was initialed by adding MUP solution (50 μL, 1 mM MUP, sodium acetate buffer, 0.1 M, pH 5.5). Fluorescence was continuously recorded as aforementioned. Activity was calculated and data is shown in **Table 1**.

MUC assay[4] was employed to measure the activity of immobilized GGG-CelA2 (CelA2-GelZyms). Re-dispersed CelA2-GelZyms (50 μL in pure water) was pipetted into 96-well MTP (black, flat bottom, GreinerBio-one). Reaction was initiated by adding MUC solution (50 μL, 1 mM MUP, potassium phosphate buffer, 0.2 M, pH 7.2). Florescence was continuously recorded as aforementioned. Activity was calculated and data is shown in **Table 1**.

BCCE assay[7] was employed to measure the activity of immobilized GGG-P450 BM3 F87A (P450 BM3 F87A-GelZyms). Re-dispersed P450 BM3 F87A-Gelzyms (50 μL in pure water) were pipetted into 96-well MTP (black, flat bottom, GreinerBio-one). Buffer (43 μL, 50 mM, pH 8.0, Tris/HCl) was added and followed by addition of BCCE (2 μL, 2 mM in DMSO). MTP plate was incubated (5 min, 800 rpm, room temperature) and the reaction was initiated by supplementing NADPH (5 μL, 10 mM). Fluorescence was constantly monitored as previously described. Activity is shown in **Table 1**.

LPETG-tagged enzymes immobilization on GGG-PVCL/GMA microgels

The immobilization of LPETG-tagged enzymes on GGG-PVCL/GMA microgels was performed similarly as aforementioned. GGG-PVCL/GMA (0.1 mg) microgels were dispersed in 240 μL buffer A (50 mM, pH 7.5, Tris/HCl) in a 1.5 mL Eppendorf reaction tube. The supernatant (150 μL) of CueO-LPETGGGRR, was pipetted into the GGG-PVCL/GMA microgels solution. Reaction was initiated by adding SrtA rM4 (10 μL, 50 μM). To immobilize P450 BM3 F87A-LPETGGGRR, the GGG-PVCL/GMA microgel (0.1 mg) was dispersed in buffer (160 μL, 50 mM, pH 7.5, Tris/HCl) and mixed with 150 μL of P450 BM3 F87A-LPETGGGRR cell free lysate. SrtA rM4 (50 μL, 40 μM) was added to initiate the conjugation. Two negative controls were performed in parallel for each immobilization experiment. In the “no tag” control, PVCL/GMA microgels (0.1 mg) were dispersed instead of GGG-PVCL/GMA microgels. In the

"no sortase" control, buffer (50 mM, pH 7.5, Tris/HCl) was added instead of SrtA rM4. The reaction mixtures were incubated (20 °C, 16 h, 800 rpm) for immobilization of CueO-LPETGGGRR or 20 °C, 16 h, 800 rpm for immobilization of P450 BM3 F87A-LPETGGGRR. After immobilization, CueO- and P450 BM3 F87A-GelZyms were washed and recovered as aforementioned.

Activity of CueO-GelZyms was measured by ABTS assay. Re-dispersed CueO-Gelzyms (25 μL in pure water) were transferred into a 96-well MTP (transparent, flat bottom, GreinerBio-one). Reaction was initiated by adding 175 μL ABTS solution (3.4 mM, pH 3.0, 100 mM, sodium citrate buffer). Plates were stirred (5 seconds) and the absorbance was continuously recorded as aforementioned. Activity of CueO-Gelzyms was calculated and data is shown in **Table 1**.

Activity of P450 BM3 F87A-Gelzyms was measured by BCCE assay. Method for the assay was performed same as aforementioned. Data of activity is shown in **Table 1.**

Quantification of the enzyme amount on PVCL/GMA microgels

Purified CueO-LPETGGGRR and GGG-P450 BM3 F87A were immobilized on GGG-PVCL/GMA and PVCL/GMA-LPETGGRR, respectively. Conditions and compounds for the immobilizations are listed in **Table S5.** GGG-PVCL/GMA microgels (0.1 mg) were dispersed in buffer (200 μL, 50 mM, pH 7.5, Tris/HCl) in a 1.5 mL Eppendorf reaction tube. Purified CueO-LPETGGGRR (130 μM, 50 mM, pH 7.5, Tris/HCl) was pipetted into the GGG-PVCL/GMA microgel suspension. $CaCl_2$ (20 μL, 100 mM), NaCl (20 μL, 3 M) and SrtA rM4 (10 μL, 50 μM) were added subsequently. The sortagging immobilization was performed by incubation (20 °C, 16 h, 800 rpm). In parallel, the "No tag" and "No sortase" controls were performed.

Likewise, the PVCL/GMA-LPETGGRR microgels (0.1 mg) were dispersed in buffer (200 μL, 50 mM, pH 8.0, Tris/HCl) in a 1.5 mL Eppendorf reaction tube. The purified GGG-P450 F87A (60 μM, in 50 mM, pH 8.0, Tris/HCl) was pipetted into the PVCL/GMA-LPETGGRR microgels suspension. SrtA rM4 (50 μL, 20 μM) was subsequently added. The sortagging immobilization was performed by incubation (20 °C, 24 h, 800 rpm). In parallel, the "No tag" and "No sortase" controls were performed.

After ligation, samples were centrifuged (15 °C, 10 min, 9390 *g*) and washed as aforementioned (see experiments of N-terminal GGG-tagged enzymes immobilization on PVCL/GMA-LPETGGRR microgels). The pellet of PVCL/GMA microgels with immobilized enzyme was re-dispersed (200 μL pure water, concentration of microgels in water is 0.5 mg/mL).

The amount of immobilized enzymes on GelZyms was quantified by BCA assay (BCA Protein Assay Kit, Novagen®, Merck Millipore, Darmstadt, Germany). In order to minimize the scattering effect of the used microgel in BCA assay, the standard curve of bovine serum albumin (BSA) was implemented by mixing different concentrations of BSA (200 μL) with relative low amount of GGG-PVCL/GMA (25 μL, 0.5 mg/mL) or PVCL/GMA-LPETGGRR (25 μL, 0.5 mg/mL). The other procedures were performed according to manufactures protocol (BCA Protein Assay Kit, Novagen®, Merck Millipore). Standard curves of BSA in GGG-PVCL/GMA or PVCL/GMA-LPETGGRR were generated (**Figure S5**). CueO-GelZyms (25 μL, 0.5 mg/mL) or P450 BM3 F87A-GelZyms (25 μL, 0.5 mg/mL) were used for BCA assay according to manufactures protocol (BCA Protein Assay Kit, Novagen®, Merck Millipore). Absorbance was measured and immobilized protein concentrations were calculated by fitting the obtained absorbance to the generated standard curves, respectively. The amounts of immobilized CueO-LPETGGGRR or GGG-P450 BM3 F87A on PVCL/GMA microgels are showed in **Table S6**.

Characterization of soluble and immobilized enzymes

Determination of the kinetic parameters of CueO and CueO-GelZyms was performed using the ABTS assay. Concentration of ABTS substrate ranged from 0.05 to 15 mM and immobilized / soluble CueO concentration was fixed at 17.8 nM. Plates were stirred for 5 seconds and the absorbance of ABTS was constantly measured. Activity was calculated and kinetic parameters were obtained by fitting activity data to the Michaelis-Menten equation (software Origin pro 8.6, OriginLab, Massachusetts, USA) (**Figure S6)**.

Characterization of P450 BM3 F87A and P450 BM3 F87A-GelZyms was performed using the BCCE assay. A standard curve for the fluorescent product 3-carboxycoumarin ethyl ester (3-CCE) was generated (**Figure S7**). Concentrations of BCCE substrate ranged from 5 μM to 200 μM and immobilized / soluble GGG-P450 BM3 F87A concentrations were fixed at 200 nM (24 μg/mL). BCCE (2 μL, 2 mM in DMSO) was subsequently pipetted into MTP and incubated (5 min, 800 rpm, room temperature). Reaction was initiated by addition of NADPH (1 μL, 10 mM). The fluorescent signal was constantly recorded as aforementioned. Activity was calculated based on the 3-CCE standard curve and kinetic parameters were obtained by fitting the activity data to the Michaelis-Menten equation (software Origin pro 8.6, Massachusetts, USA) (**Figure S8**).

Activity profiles of free and immobilized enzymes in organic solvents

The effect of solvents on immobilized and soluble CueO was studied. Activity of CueO-LPETGGGRR and CueO-GelZyms in different concentrations of DMSO co-solvent (ranging from 0 to 50%) was investigated. ABTS assay was initiated by incubated with 17.8 nM (1 μg/mL) of CueO-LPETGGGRR enzyme or CueO-Gelzyms. Protocols of ABTS assays were performed same as aforementioned (see CueO characterization experiment).

In order to investigate the resistance of P450 BM3 F87A-Gelzyms in organic co-solvent, activity of soluble and P450 BM3 F87A-Gelzyms in gradient DMSO co-solvent was studied. The concentrations of DMSO were implemented from 0 to 25% (v/v) with 200 nM (24 μg/mL) soluble or P450 BM3 F87A-GelZyms in BCCE solution (100 μL, 40 nM, pH 8.0, Tris/HCl) followed by incubation (5 min, 800 rpm, room temperature). The reaction was initiated by addition of NADPH (1 μL 10 mM). Fluorescence was constantly recorded as described above.

Activity profiles of soluble and immobilized enzymes at different pH values

Sodium citrate buffer (pH range from 2.6 to 5.0) was employed as the buffer for pH profiles of CueO laccase. In detail, 36.6 nM soluble or CueO-Gelzyms were incubated in ABTS solution (200 μL, 3 mM ABTS, 100 mM, sodium citrate buffer) with different pH values in MTP (transparent, flat bottom) and the absorbance was continuously recorded.

The activity profile of P450 BM3 F87A at different pH values was measured by BCCE assay. In detail, 0.2 μM soluble or P450 BM3 F87A-GelZyms were incubated in BCCE solution (100 μL, 44.5 nM BCCE, 100 mM, potassium phosphate) in different pH (range from 5.7 to 8.6) in MTP (black, flat bottom). Sample was incubated (5 min, 800 rpm, room temperature). The reaction was initiated by adding NADPH (1μL, 10 mM) and fluorescence was constantly recorded.

Storage stability of immobilized enzymes

The storage stability of CueO-GelZyms and P450 BM3 F87A-GelZyms was implemented by monitoring their activity when stored at 4 °C. CueO and CueO-GelZyms were incubated (1.25 μM CueO, 4 °C, 50 mM, pH 7.5, Tris/HCl buffer). For activity measurement, aliquots (5 μL) were pipetted every seven-day into ABTS solution (195 μL, 3 mM ABTS, sodium citrate buffer, pH 3.0). Absorbance of ABTS was measured as aforementioned.

Samples of soluble P450 BM3 F87A and P450 BM3 F87A-GelZyms were incubated in buffer (2 μM enzymes, 4 °C, 0.05 M, pH 8.0, Tris/HCl buffer). At different time points (ever 24 h), aliquot (10 μL) of incubated samples were pipetted into 83 μL BCCE solution (48.2 μM BCCE, 50 mM, pH 8.0, Tris/HCl) in MTP (black, flat bottom). After incubation (5 min, 800 rpm, room temperature), reaction was initiated by addition of NADPH (1 μL, 10 mM). The fluorescence was constantly recorded as aforementioned.

Reusability of CueO- and P450 BM3 F87A-GelZyms

CueO-GelZyms (0.1 mg) were dispersed and incubated (2 min, 800 rpm, room temperature) in ABTS solution (200 μL, 3 mM ABTS in 100 mM, pH 5.5 sodium acetate) in a 1.5 mL Eppendorf reaction tube. Sample was then centrifuged (3 min, 9390 *g*, 15 °C). Supernatant was immediately transferred to MTP (transparent, flat bottom) and the absorbance was recoded as aforementioned. The CueO-Gelzyms pellet was re-dispersed and incubated (2 min, 800 rpm, room temperature) in ABTS solution (200 μL, 3 mM ABTS in 100 mM, pH 5.5 sodium acetate) for absorbance detection in cycle 2. Ten consecutive cycles were performed with the same protocol.

Reusability tests of P450 BM3 F87A-GelZyms were carried out by dispersing P450 BM3 F87A-Gelzyms (0.25 mg) in BCCE solution (100 μL, 80 μM BCCE, 50 mM, pH 8.0, Tris/HCl) in MTP (black, flat bottom). The sample was firstly incubated (5 min, 800 rpm, room temperature) and then the reaction was initiated by adding NADPH (1μL, 20 mM). Fluorescence was constantly recorded as aforementioned. In the second cycle, the MTP plate containing PVCL/GMA-LPETGGG-P450 F87A was centrifuged (Eppendorf centrifuge 5810 R, 3220 g, 30 min, 4°C). The supernatant was pipetted out. Fresh BCCE solution (40 μM BCCE in 50 mM, pH 8.0, Tris/HCl) was added, the pellet was dispersed, and the fluorescence was recorded. Six consecutive cycles were implemented using the same protocol.

Application of CueO-GelZyms in decolorization of indigo carmine

Indigo carmine was selected as the dye to be bleached using the CueO-GelZyms as the catalyst. In brief, 0.25 mM indigo carmine, 1.5 mM $CuSO_4$ (CueO shows enhanced oxidase activity when additional Cu^{2+} is present),[10] and the CueO-GelZyms (0.263 mg, containing 17 μg CueO laccase) were incubated (1000 rpm, room temperature) in buffer (200 μL, 0.1 M, pH 5.5, sodium acetate) in a 1.5 mL Eppendorf reaction tube. Three controls were performed in parallel. In control 1, GGG-PVCL/GMA microgels (0.263 mg) were added instead of CueO-GelZyms. In control 2, 17 μg of soluble CueO-LPETGGGRR laccase were added instead of CueO-GelZyms. In control 3, no CueO-GelZyms but sodium acetate buffer was supplemented. The sample was centrifuged (10 min, 9390 *g*, 15 °C). The supernatant was transferred into transparent 96-well MTP well. The absorbance of the supernatant was measured at 608 nm after 1, 16, 40, 120 and 180 h incubation. Data is shown as percentage of decreased color absorbance relative to the absorbance in control 3.

Reusability tests of CueO-GelZyms in decolorization of indigo carmine were carried out based on the protocol described above. In brief, 0.25 mM indigo carmine, 1.5 mM $CuSO_4$, and CueO-Gelzyms (0.4 mg, containing 26 μg CueO laccase) were incubated (1000 rpm, room 20 °C) in buffer (200 μL, 0.1 M, pH 5.5, sodium acetate) in a 1.5 mL Eppendorf reaction tube. After 24 h, the sample was centrifuged (3 min, 9390 *g*, 15 °C). The supernatant was transferred into MTP (transparent, flat bottom) and the absorbance was measured at 608 nm. The pellet of CueO-GelZyms in the reaction tube was dispersed again with fresh dye solution (200 μL, 0.25 mM indigo carmine, 1.5 mM $CuSO_4$, 0.1 M, pH 5.5, sodium acetate buffer) in cycle 2. The same protocol was used in each reuse cycle. One control of reusability was performed in parallel in which GGG-PVCL/GMA microgels (0.4 mg) instead of CueO-GelZyms were added.

List of Tables

Table S1. List of primers used for the gene construction of N-terminal GGG and C-terminal LPETGGGRR tagged enzymes

Primer Name	**Sequence 5′-3′**
Fw ggg-his-bsla	GGTGGAGGACATCATCATCATCATCATGATATAGCTGAAC
Rev ggg-his-bsla	ATGATGATGATGATGATGTCCTCCACCGGCCATCGCCGG
Fw ggg-phytase	GGAGGAGGACGATTAACTGCACTGGGCTTAAT
Rev ggg-phytase	TCCTCCTCCCATATGTATATCTCCTTCTTAAA
Fw ggg-cueo	ATGGGTGGCGGGGCAGAACGCCCA
Rev ggg-cueo	CCCGCCACCCATATGTATATCTCC
Fw ggg-cel-a2	ACCATGGGTGGTGGTAGCAGCCATCACCAC
Rev ggg-cel-a2	ACCACCACCCATGGTATATCTCCTTCTTAA
Fw ggg-his-p450	GGAGGAGGACATCATCATCATCATCATACAATTAAAGAAATG
Rev ggg-his-p450	ATGATGATGATGATGATGTCCTCCTCCCATGCTGCCCAGGGT
Fw p450 lpetgggrr	CTACCTGAAACAGGTGGTGGTCGTCGTTAAGCTAACAAAGCCCGA
Rev p450 lpetgggrr	ACGACGACCACCACCTGTTTCAGGTAGCCCAGCCCACACGTC

Table S2. Theoretical molecular weights (Mw), isoelectric points (pI) and detected Zeta potentials (in 50 mM, pH 7.5, Tris/HCl buffer) of GGG- and LPETG- tagged enzymes

Enzyme	**Molecular weight (kDa)**	**Isoelectric point (pH)**	**ζ-potential (mV)**
GGG-BSLA	20.7	9.05	Not detected
GGG-Ym-phytase	47.5	6.59	Not detected
CueO-LPETGGGRR	55.9	6.1	-18.80 ± 4.56
GGG-CelA2 M2	71.7	4.45	-11.31 ± 2.78
GGG-P450 BM3 F87A	118.8	5.34	-7.83 ± 2.21
P450 BM3-LPETGGGRR F87A	119.6	5.37	Not detected

Table S3. Hydrodynamic radii, PDI values and Zeta-Potentials of the PVCL/GMA and peptide-tagged microgels

	PVCL/GMA*	**PVCL/GMA-LPETGGRR***	**PVCL/GMA****	**GGG-PVCL/GMA****
Hydrodyn. Radius [nm]	253.80 ± 4.11*	276.5 ± 1.09*	273.80 ± 0.50	308.7 ± 13.1
PDI	0.038*	0.061*	0.067	0.453
ζ-potential (pH 7.5) [mV]	-2.82 ± 1.12*	9.70 ± 3.01*	Not detected	-0.82± 0.46

*Previously synthesized batch.[9]
** Synthesized batch in this work.

Table S4. List of compounds and conditions for N- or C-terminal sortagging of enzymes on peptide-tagged PVCL/GMA microgels

Approach	Enzyme	Lysate (μL)	GGG-PVCL/GMA (mg/mL)*	PVCL/GMA-LPETGGRR (mg/mL)*	$CaCl_2$ (mM)	NaCl (mM)	SrtA rM4** (μM)	Temp (°C)	Time (h)
N-terminal enzyme immobilization	GGG-BSLA	150		0.25	5	150	1.25	20	16
	GGG-Ym-phytase	150		0.25	5	150	1.25	20	16
	GGG-CelA2	150		0.25	5	150	1.25	20	16
	GGG-P450 BM3 F87A	150		0.25			5	20	24
C-terminal enzyme immobilization	CueO-LPETGGGRR	150	0.25		5	150	1.25	20	16
	P450 BM3 F87A-LPETGGGRR	150	0.25				5	20	24

* Concentrations were calculated as dried weight of microgels

**Sa-SrtA rM4: P94S/D160N/D165A/K196T[8]

Table S5. Reaction settings of sortagging using purified enzymes as substrates

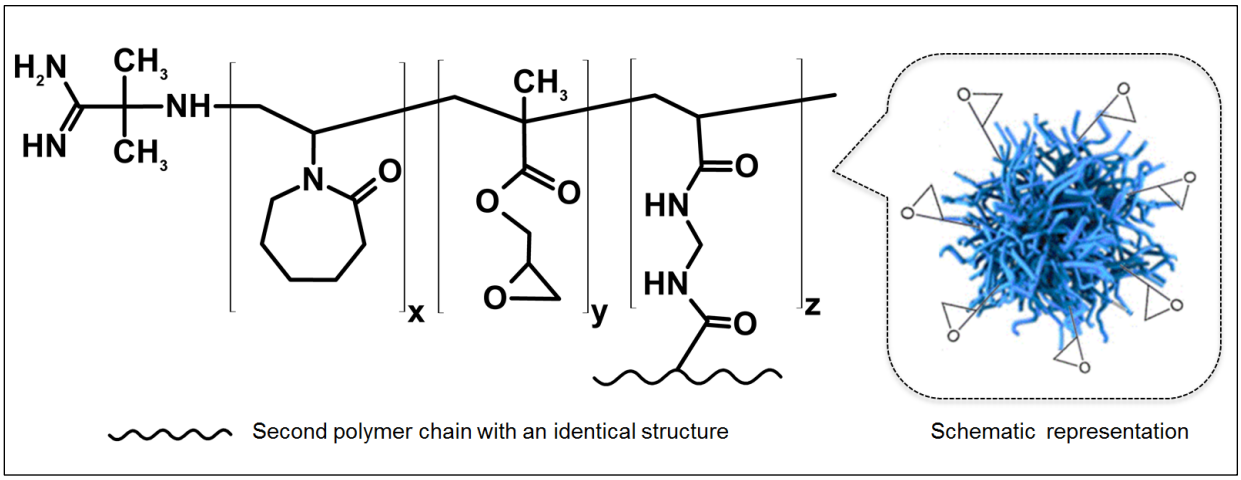
	Enzyme (μM)	GGG-PVCL/GMA (mg/mL)*	PVCL/GMA-LPETGGRR mg/mL)*	$CaCl_2$ (mM)	NaCl (mM)	SrtA rM4 (μM)	T (°C)	Time (h)
C-terminal CueO-LPETGGGRR sortagging	50	0.25		5	150	1.25	20	16
C-terminal GGG-P450 F87A sortagging	50		0.25			2.5	20	24

* Concentrations were calculated as dried weight of microgels

Table S6. Immobilized protein amounts in microgels

Ligation	CueO amount in microgels (μg /per mg dried microgels)	P450 BM3 (F87A) amount in microgels (μg /per mg dried microgels)
No tag control	11.4 ± 1.9	20.8 ± 2.5
No sortase control	13.2 ± 2.1	Not detected
Sortase-ligated enzyme sample	64.2 ± 8.2	89.6 ± 6.2

List of Figures



Scheme 1. Structure of PVCL/GMA microgels

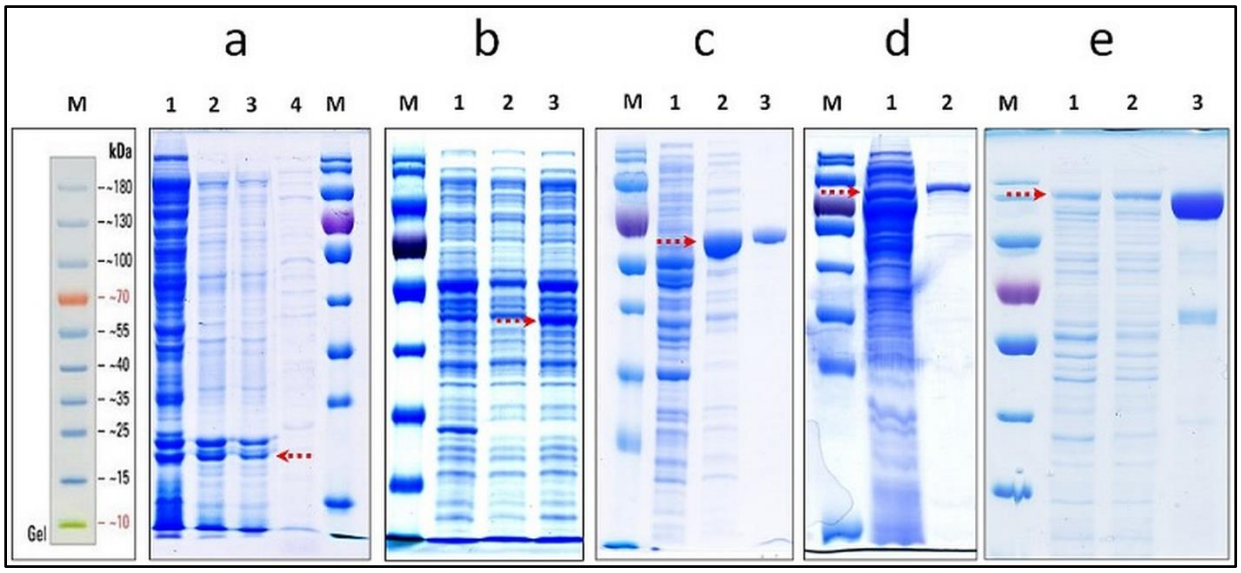


Figure S1. Expression of GGG- and LPETG-tagged enzymes. a) Expression of GGG-BSLA. Lane 1: supernatant of GGG-BSLA cell lysate; lanes 2-4, elution fractions of GGG-BSLA during His-tag based purification. Expected size of GGG-BSLA is 20.7 kDa. b) Expression of GGG-Ym-phytase. Lane 1: cell lysate of *E.coli* harboring pET-22b (+) empty vector; lane 2: pellet of Ym-phytase lysate; lane 3: supernatant of GGG-Ym-phytase lysate. Expected size of GGG-Ym-phytase is 47.5 kDa. c) Expression of CueO-LPETGGGRR. Lane 1: cell lysate of *E.coli* harboring pET-22b(+) empty vector; lane 2: supernatant of CueO-LPETGGGRR cell lysate; lane 3: purified CueO-LPETGGGRR. Expected size of CueO-LPETGGGRR is 55.9 kDa. d) Expression of GGG-CelA2 M2. Lane 1: supernatant of GGG-CelA2 M2 cell-free lysate; lane 2: purified GGG-CelA2 M2. Expected size of GGG-CelA2 M2 is 71.5 kDa. e) Expression of GGG-P450 BM3 F87A and P450 BM3 F87A -LPETGGGRR. Lane 1: supernatant of GGG-P450 F87A cell-free lysate; lane 2: supernatant of P450 BM3-LPETGGGRR F87A cell-free lysate; lane 3: purified GGG-P450 BM3 F87A. Expected sizes of GGG-P450 BM3 F87A and P450 BM3-LPETGGGRR F87A are 118.9 and 119.6 kDa, respectively.

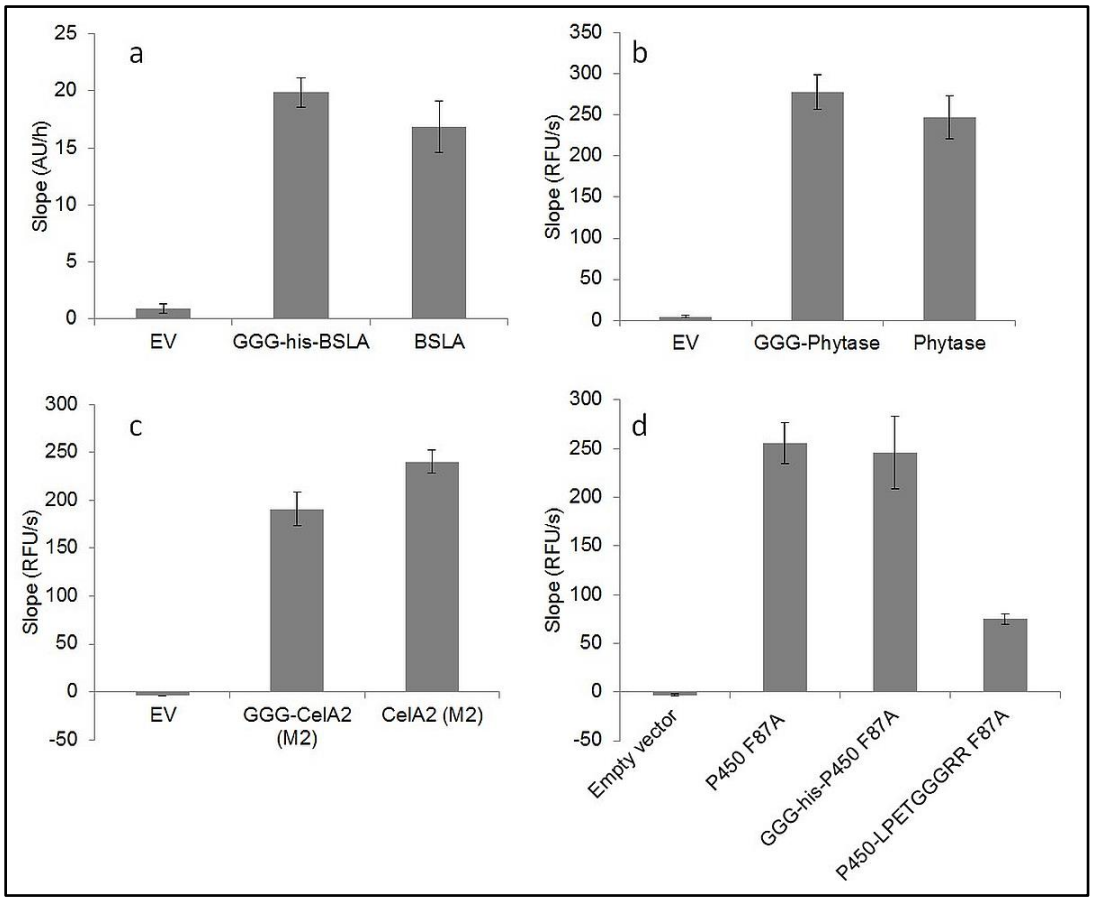


Figure S2. Activity of GGG- and LPETG-tagged enzymes in comparison to non-tagged enzymes. a) Activity of *Bacillus subtilis* lipase A (BSLA) in pNPB assay; b) Activity of *Yersinia mollaretii* phytase (Ym-phytase) in 4-MUP assay; c) Activity of cellulase A2 M2 variant (CelA2 M2) in 4-MUP assay; d) Activity of monooxygenase P450-BM3 F87A variants in BCCE assay. All assays were performed with enzymes in cell-free lysate. Protocols are described as aforementioned.

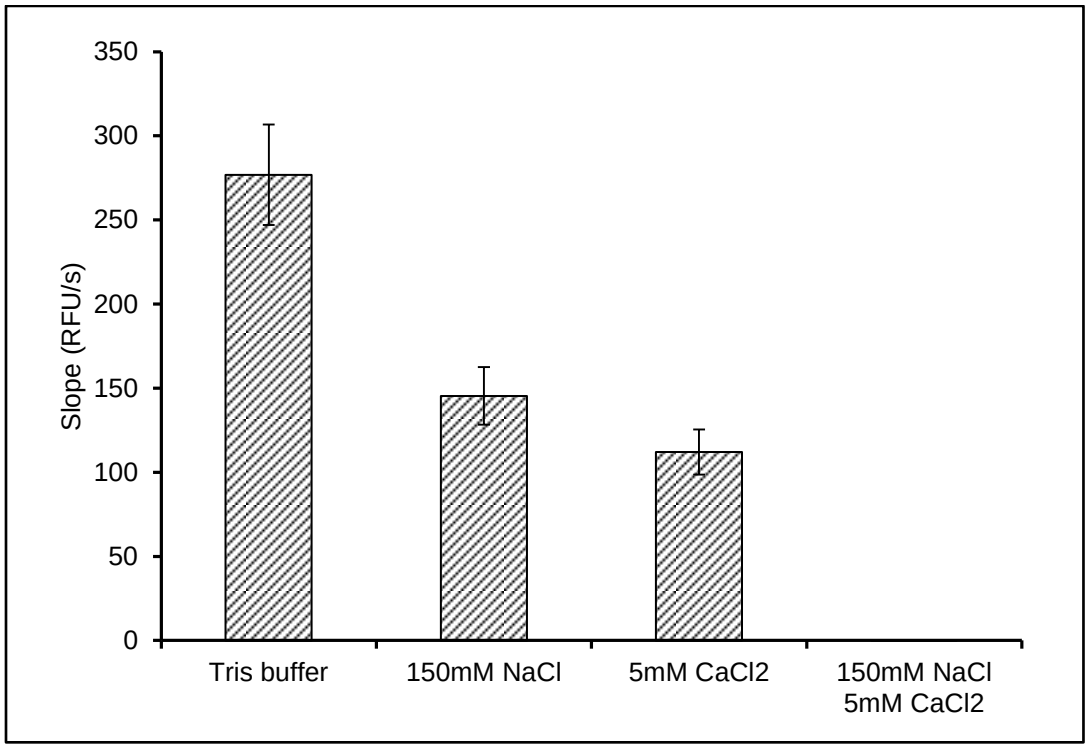


Figure S3. Activity of GGG-P450 F87A in salt conditions. Supernatant of GGG-P450 F87A cell lysate was used in BCCE assay. All salts were incubated in Tris/HCl buffer (50 mM, pH 8.0).

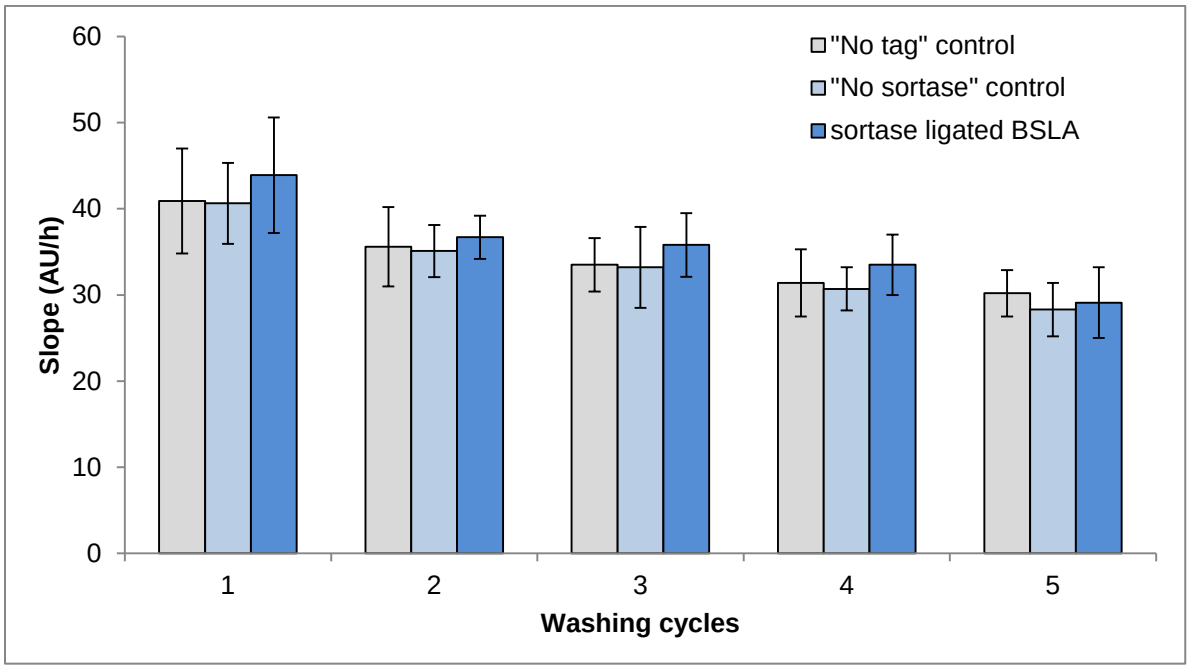


Figure S4. Optimization of washing steps in immobilization of GGG-BSLA on PVCL/GMA-LPETGGRR microgels. After ligation, microgels were recovered by centrifugation. The gel pellet was subsequently dispersed, washed and centrifuged in consecutive cycles. Activities of washed microgels were measured with pNPB assay after each cycle. Protocols of washing and pNPB assay are described as aforementioned.

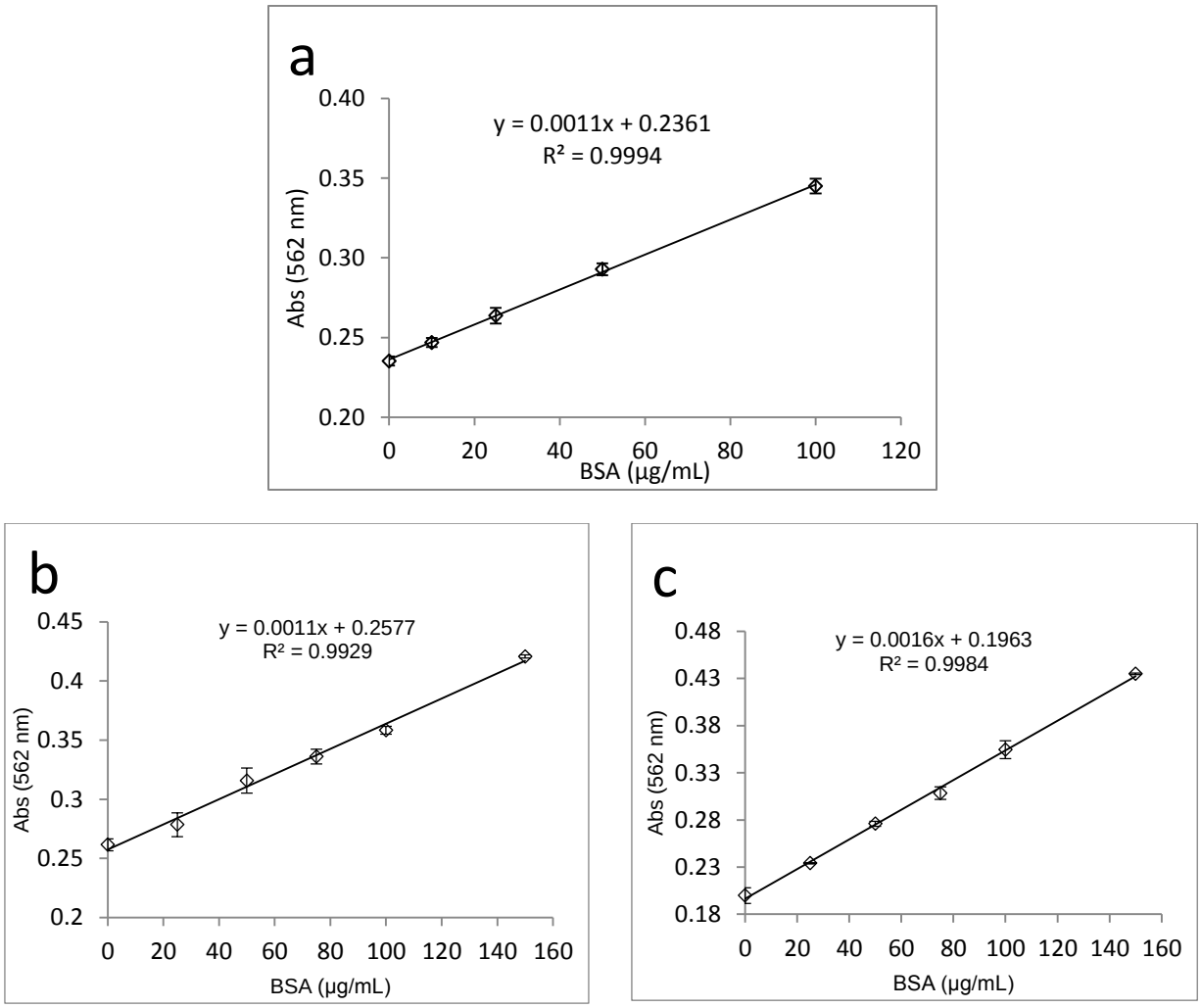


Figure S5. Standard curves of concentration of BSA in microgel solutions. a) BSA was incubated with 0.5 mg/mL PVCL/GMA microgels; b) BSA was incubated with 0.5 mg/mL GGG-PVCL/GMA microgels; c) BSA was incubated with 0.5 mg/mL PVCL/GMA-LPETGGGRR microgels. BCA assays were perfomed accroding to the manufacture's protocol.

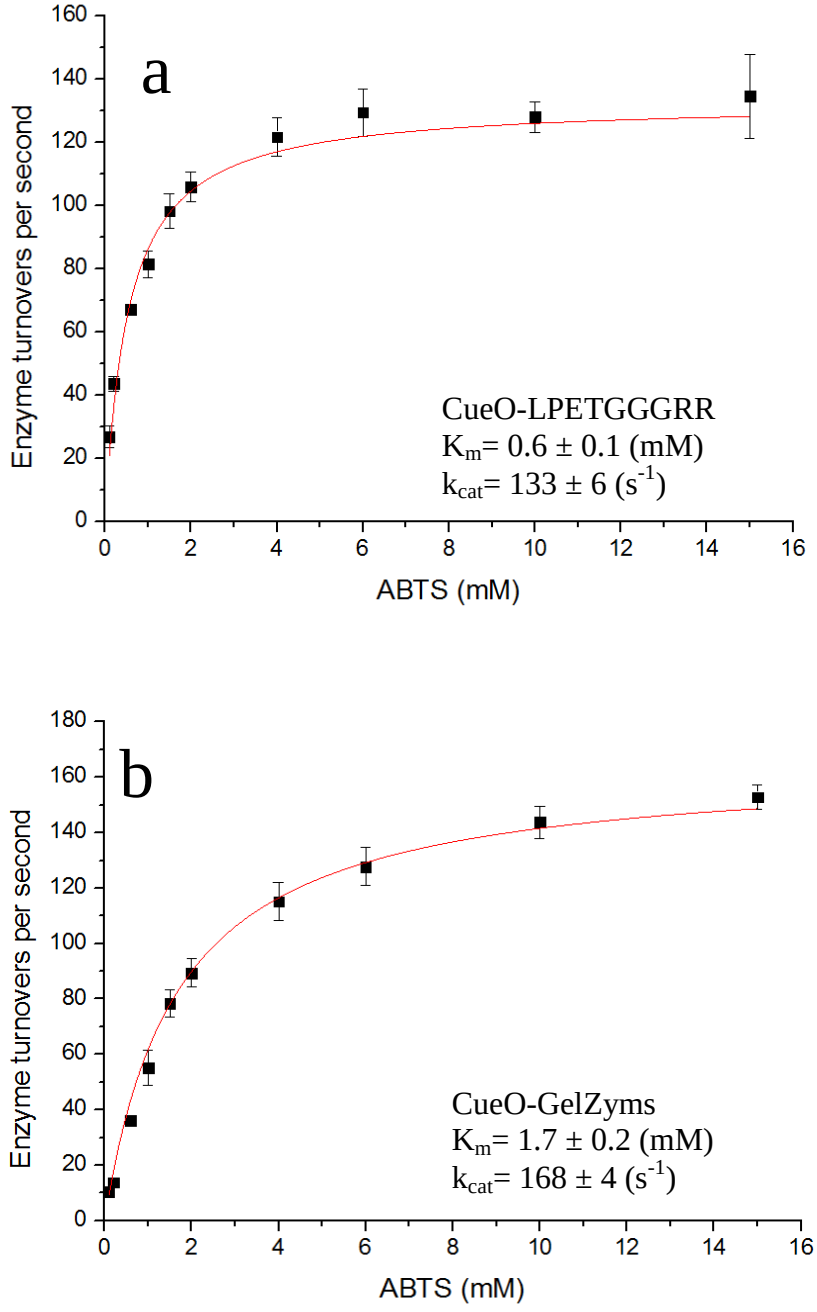


Figure S6. Plots to determine the kinetics (K_m and k_{cat}) of CueO-LPETGGGRR (a) and CueO-GelZyms (b).

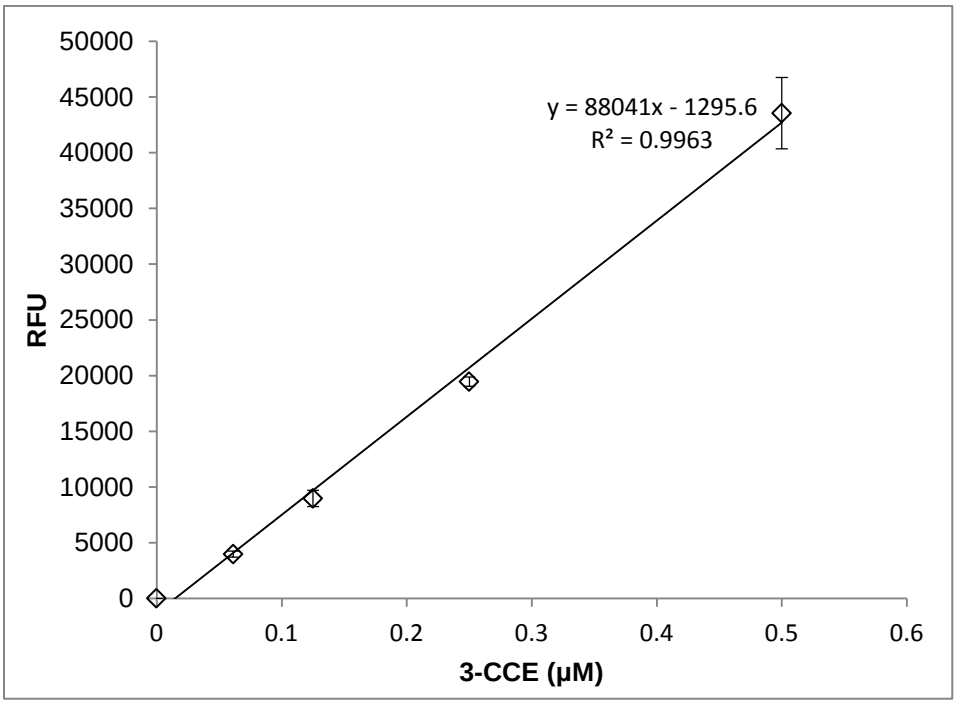


Figure S7. Relative fluorescence unit of 3-CCE related to its concentrations

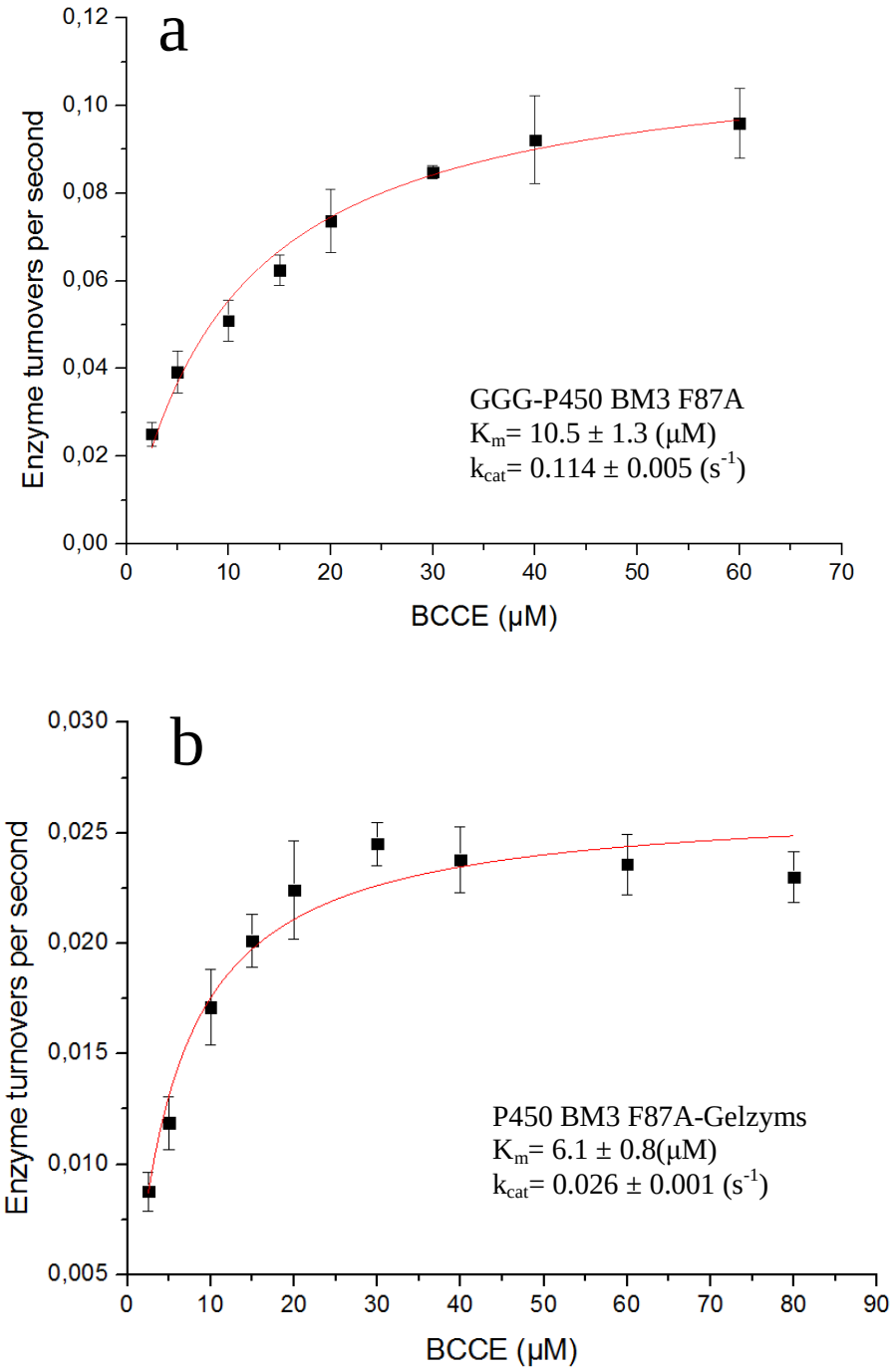


Figure S8. Plots to determine the kinetics (K_m and k_{cat}) of GGG-P450 BM3 F87A (a) and P450 BM3 F87A-GelZyms (b).

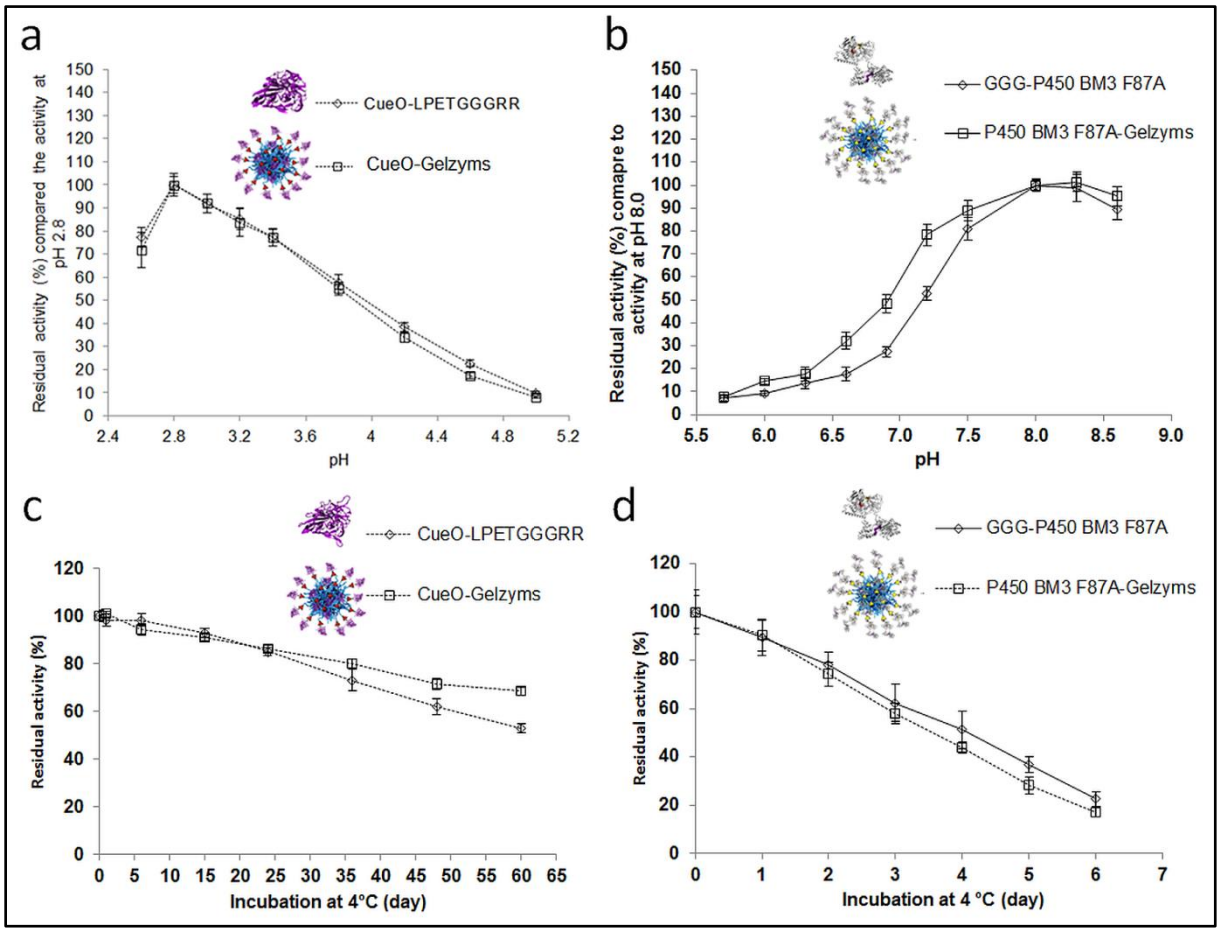


Figure S9. pH activity and storage stability profiles of CueO-GelZyms and P450 BM3 F87A-GelZyms. a) pH activity of soluble CueO and CueO-Gelzyms; b) pH activity of soluble P450 BM3 F87A and P450 BM3 F87A-Gelzyms; c) Storage stability of soluble CueO and CueO-Gelzyms at 4 ^{o}C; d) Storage stability of P450 BM3 F87A and P450 BM3 F87A-Gelzyms at 4 ^{o}C.

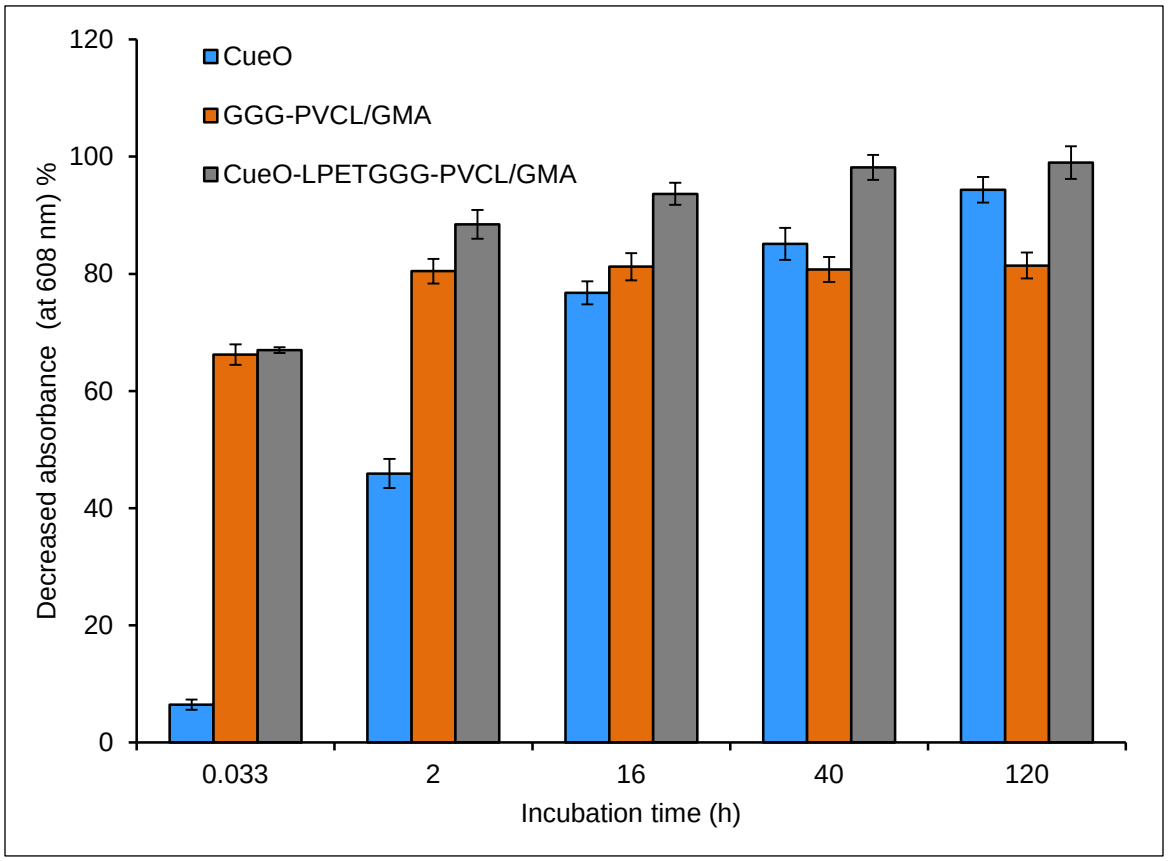


Figure S10. Time-dependent decolorization of indigo carmine by CueO, GGG-PVCL/GMA microgels, and CueO-Gelzyms.

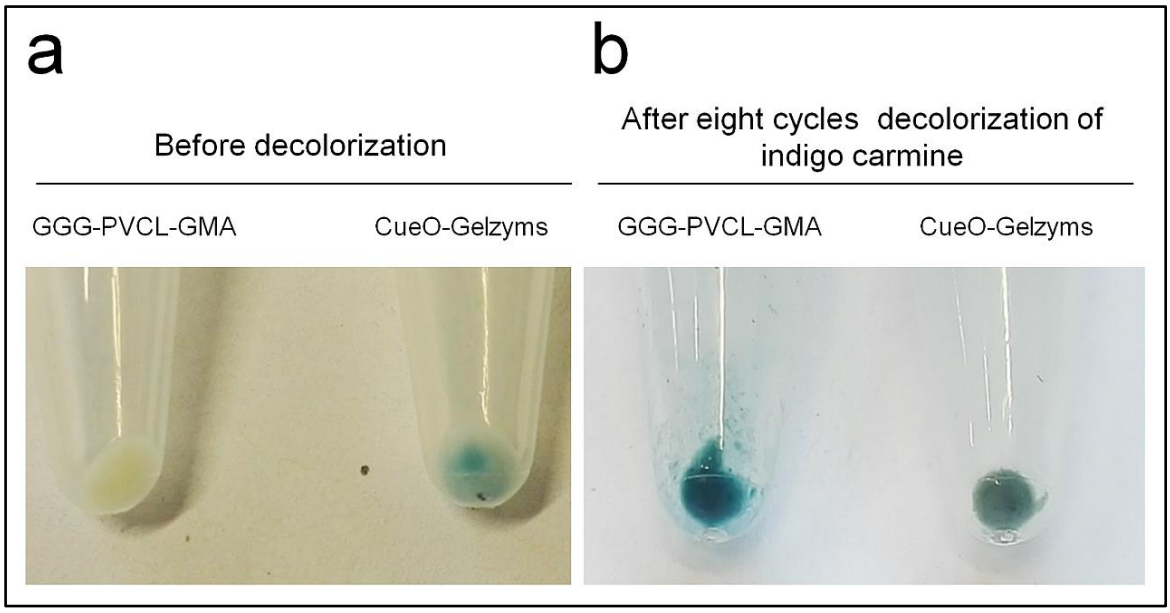


Figure S11. Photos of microgels (GGG-PVCL/GMA and CueO-GelZyms before (a) and after (b) using in decolorization of indigo carmine.

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