Microgel-Engineered Temperature-Responsive Microcapsules at Liquid Interfaces for Sequential Biocatalytic Reaction

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S1. General methods

UV-visible spectroscopy was performed on a temperature variant UV-vis spectrophotometer to determine the LCST of the microgels. The aqueous dispersion of microgels was analysed at wavelength 290 nm and the temperature was varied from 24 to 40 °C at a heating rate of 2.0 °C/min.

Transmission electron microscopy (TEM) analysis was performed on a JEOL-1400 TEM to analyze the internal morphology and particle size of the PNSER core-shell microgel particles. The microgel samples were prepared using the drop-casting method. An aqueous dispersion of microgels (0.001 mg/ mL) was drop-casted onto a carbon-coated copper grid and dried at room temperature for 24 h in anhydrous conditions.

Field emission scanning electron microscopy was performed to study the surface morphology and topography of the PNSER microgels and derived microgelsomes using (FE-SEM, JSM-7800F Prime, JEOL instrument. The aqueous dispersion of samples was drop-casted on a clean glass slide and air-dried under anhydrous conditions. The specimens were sputter coated with 10 nm platinum and characterized at 5kV accelerating voltage.

Atomic force microscopy (AFM) images of microgels were performed on a Bruker multimode instrument with a Quadrexed Nanoscope 3D controller. Samples were drop-casted onto a silica wafer and air-dried overnight.

Confocal laser scanning microscopy (CLSM) was performed to analyse microgelsomes formation, their particle size, stability and encapsulation of guest molecules using Leica Microsystems, Germany at 100x magnification. Samples were prepared by adding a 5 µL dispersion of freshly prepared microgelsomes on a clean glass slide and covered with a glass coverslip for microscopic analysis.

Dynamic light scattering (DLS) was used to characterize the hydrodynamic sizes at different temperatures and their surface charge at different pH values, using Zeta sizer Ver. 7.11., Malvern Instruments, UK.

Interfacial tension between the microgel-rich aqueous phase and 2-ethyl 1-hexanol was measured using the du Noüy ring method on a manual tensiometer (Testing instruments S1 manufacturing company). The aqueous microgel dispersion was filled in a glass apparatus, and the du Noüy ring was immersed 4mm below the surface, followed by the careful addition of 2-ethyl 1-hexanol. The du Noüy ring was then slowly lifted upwards until the lamella tore off the ring and the interfacial tension was measured.

Reverse phase high-performance liquid chromatography (HPLC) was used to examine the quantitative analysis of acetophenone and (S)-α-methylbenzylamine (S-MBA) from 1-phenyl alcohol at 210 nm using a C-18 symmetry column (250 mm × 4.6 mm × 5μm, Shimadzu Japan) with a flow rate of 1 mL/min for 10 minutes. The sample injection volume was 10 μL. The column temperature was constant at 25 °C and the photodiode array (PDA) detector was used. Quantitative analysis was carried out using peak areas, which were calibrated against external standards. GC-MS analysis was done for further confirmation.

S2. Experimental Section

S2.1. Materials

N-isopropylacrylamide (97%; NIPAM) recrystallized in *n*-hexane, rhodamine 6G (Rh6G), ferric chloride ($FeCl_3$), *N,N'*-methylene bisacrylamide (BIS), copper(II) carbonate basic ($CuCO_3.Cu(OH)_2$), acryloyl chloride (≥ 97 %), and 8-hydroxyquinoline were purchased from TCI. Ammonium persulfate (APS), L-serine, 2,2,6,6-tetramethylpiperidinoxyl radical (TEMPO), poly(propylene glycol) diglycidyl ether (PPGDGE, M_W = 640 g/mol) 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), laccase from *Tramates versicolor*, 2-ethyl hexanol, FITC-dextran (FD; 20 kDa), fluorescein isothiocyanate (FITC), rhodamine isothiocyanate (RITC), acetophenone, 1-phenyl ethanol, alanine, (S)-α-methylbenzylamine (S-MBA) were purchased from Sigma-Aldrich, USA.

S2.2. Synthesis of *O*-acryloyl L-serine

The synthesis of *O*-acryloyl L-serine (Ser-Ac) was performed as per the method reported in previous literature[1] and shown in **Figure S1**. Briefly, L-serine (48 mmol) was first dissolved in 50 mL water maintained at 90 °C, followed by the slow addition of 27 mmol basic cupric carbonate with continuous stirring. The reaction was continued for 15 min. The insoluble residue was filtered and washed with 30 mL of hot water. To this copper complex of L-serine, 10 mL of acetone and 30 mL of 2M KOH were added, and the reaction mixture was cooled to 0 °C with subsequent dropwise addition of 60 mmol acryloyl chloride. The reaction was constantly stirred for 12 h at room temperature, resulting in the precipitation of a blue-colored complex of acryloyl cupric complex. The precipitate was filtered and washed first with water, and then with methanol and diethyl ether.

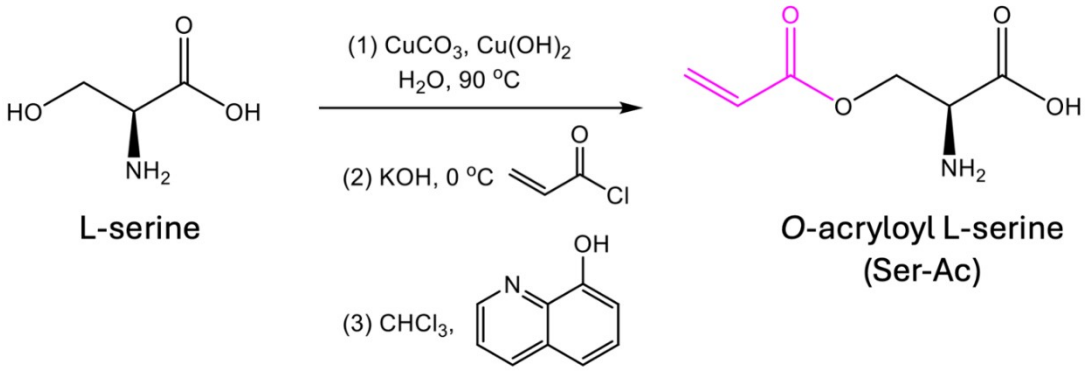

Figure S1. Synthesis scheme for monomer Ser-Ac by *O*-acryloylation of L-serine.

Further, the solid acryloyl cupric complex dispersed in 300 mL water was mixed with 300 mL chloroform solution containing 55 mmol 8-quinolinol. The reaction mixture was shaken for 12 h in the separating funnel which separated the green-colored copper-hydroxy quinolinol complex in the organic phase and was filtered off. The residual 8-quinolinol was removed by subsequent washing with chloroform. The aqueous layer was concentrated to 50 mL and lyophilized. The white solid obtained was recrystallized from tetrahydrofuran.

The structure of Ser-Ac was confirmed using ^{1}H NMR (500 MHz, D_2O) **(Figure S3 (A), ESI)**: The ^{1}H d (ppm): 3.23 (d, 1H, CH), 3,81 (m, 2H, CH_2), 4.83 (t, 2H, CH_2), 7.32 (d, 1H, CH_2= CH (trans)), 7.81 (dd, 1H, CH_2=CH), 8.01 (d, 1H, CH_2= CH (cis)), FTIR (cm^{-1}) **(Figure S3 (B), ESI** 3352, ν_{N-H} (amide); 2875–2965, ν_{C-H}; 2210-3220, ν_{N-H} (NH_3^+); 2100-2225, δ_{N-H} (NH_3^+); 1667, $\nu_{C=O}$ (amide I); 1625, δ_{N-H} (amide II).

S2.3. Synthesis of the PNSER core-shell microgel

The core-shell PNSER microgel particles were synthesized using the free radical coprecipitation method in two steps. Stepwise representation for the synthesis of PNSER microgel. Step 1 shows the chemical route to the synthesis of PNIPAM nanoparticles which serve as seed particles for the Step 2 reaction: the polymerization of monomer Ser-Ac on PNIPAM particles, resulting in the formation of PNSER microgels with core-shell morphology. In the first step, monomers NIPAM and varying amounts of BIS, and SDS (2 mM) dissolved in 75 mL DI water were added to a three-neck round bottom flask equipped with a N_2 inlet, a reflux condenser, and a thermometer. The components of the polymerization reaction are outlined in **Table S1.** The reaction mixture uniformly stirred at 300 rpm was purged with a gentle stream of N_2 for 2 h. Upon temperature equilibration to 70 °C, 1 mL of APS (2 mM) was added dropwise to the reaction solution to initiate copolymerization. The reaction was continued for 4 h at 70 °C at a continuous stirring rate. Subsequently, the solution

was cooled to room temperature and filtered using a 0.45 μm syringe filter prior to the second step, i.e., shell formation.

Table S1. Amount of different reaction components used in the synthesis of PNSER microgel particles.

PNSER microgel	NIPAM (mg)	Ser-Ac (mg)	BIS (mg)	BIS (mol%)
PNSER_10	800	200	10	0.93
PNSER_15	800	200	15	1.39
PNSER_20	800	200	20	1.85

For the synthesis of the shell: 20 mL of core solution was dispersed in 55 mL DI water containing monomer SER-Ac (17 mM) and SDS (0.7 mM) and purged with N_2 for 2 h under continuous stirring. The temperature was equilibrated to 70 °C and subsequently, APS solution (1.5 mM) was added to initiate the reaction. The reaction continued for 5 h under continuous stirring and an inert atmosphere. Upon completion, the core-shell microgel solution was filtered using a 0.45 μm syringe filter and dialyzed using a 10 kDa MWCO dialysis tube for a week against DI water with constant change after 24 h to remove unreacted monomers and low molecular weight polymer particles.

To verify the copolymerization and successful formation of PNSER microgels, ^{1}H NMR spectroscopy was carried out, with the resulting spectrum shown in **Figure S3**. The absence of vinylic signals across the spectra confirmed the completion of the reaction and the purity of the synthesized microgels. Characteristic broad peaks observed at 1.02 ppm and 3.5 ppm correspond to the methyl and methine protons of the isopropyl moiety in PNIPAM.[2] The presence of serine within the microgel was indicated by signals at 4.01 and 3.75 ppm, which are attributed to the α and β methylene protons in the serine backbone. Further evidence came from broad peaks at 1.43 and 1.8 ppm, assigned to the secondary and tertiary carbon protons of the polymer backbone, respectively.

S2.4. Preparation of microgelsomes

The synthesized PNSER microgel were assembled on both W/O and O/W emulsion droplets which were then interlinked to obtain stable microgelsomes. W/O microgelsomes were constructed as the previously reported method with some modifications.[3] Briefly, 1 mL of 2-

ethyl-1-hexanol was added to 0.3 mL aqueous dispersion of PNSER microgel particles (2 mg/mL, pH 5) and 50 µL of $FeCl_3$ solution (20 mg/mL). The mixture was vortexed at 2000 rpm for 60 s, and the resulting emulsion was incubated at room temperature for 1 h to allow the complete interlinking of microgels at the W/O interface (via amine-Fe^{+3} chemistry). The microgelsomes obtained were washed thrice with water to remove free microgels. Upon the addition of water, the less dense microgelsomes form a layer at the oil-water interface, separating the free microgel constituting the aqueous layer from the upper oil phase. The bottom aqueous layer was then injected out, followed by the careful removal of the oil phase. The residual oil from microgelsomes was removed completely by first dialyzing against a mixture of ethanol and water (50:50) for 12 h and finally against water for 12 h, for complete phase transfer. The purified microgelsomes were stored in an aqueous solution for further use and characterization.

For the preparation of microgelsomes with hydrophobic lumen, O/W emulsion droplets were used as templates onto which PNSER microgels were spontaneously assembled. Briefly, 0.3 mL PNSER microgel dispersion in 2-ethyl 1-hexanol (2 mg/ mL) containing PPGDGE (1 wt % of the microgel mass), was mixed with 1 mL DI water. The mixture was vortexed at 2000 rpm for 2 min, followed by incubation for 1 h at 30 °C to allow complete interlinking (amine-epoxy reaction) of the microgels at the O/W interface.

S2.5. Expression and purification of STH

The STH gene was codon optimized for *Escherichia coli* usages and synthesized by Genscript, USA. The gene was cloned in pET 21a(+) using NdeI and XhoI restriction sites. STH was overexpressed in *E.coli* (BL21) cells and purified using the Ni-NTA column against the imidazole gradient **(Figure S4).**

S2.6. Encapsulation and membrane permeability of microgelsomes

The encapsulation of molecules in PNSER microgel-derived microgelsomes was studied by encapsulating molecules with varying molecular weight (*Mw*) (Rh6G; M_w = 479 Da, FITC-dextran; M_w = 20 kDa) in the microgelsomes. The encapsulation of the molecules was performed during the formation of microgelsomes. Briefly, individual samples containing 100 µL of neutral FITC-dextran aqueous solution (10 mg/mL), 100 µL of Rh6G (10 mg/mL), 100 µL of STH (12 mg/mL), and 100 µL of laccase (10 mg/mL) was mixed with 200 mL microgel solution (5 mg/mL). To this, 1 mL of 2-ethyl 1-hexanol and 50 µL of $FeCl_3$ solution (20 mg/mL) were added. The mixture was vortexed for 60 s at 2000 rpm and was left undisturbed

at room temperature for 1 h. The microgelsomes so formed were purified using the method described in **Section S2.4.**

Vitamin E was used as a model lipophilic bioactive compound to test the efficacy of microgelsomes prepared via O/W Pickering emulsion. First, vitamin E was dissolved in the microgel oil dispersion (5 mg/mL) at the concentration of 10 % (w/w) and then emulsified with water. For imaging the microgelsomes using confocal laser scanning microscopy (CLSM), the enzymes labelled with fluorescent dyes; STH with dye FITC and laccase with dye RITC were used **(Section S2.8).**

The change in membrane permeability of microgelsomes with temperature was studied at varying temperatures from 20 – 50 °C. The samples were equilibrated for 10 min before measuring the absorbance at wavelength 530 nm for Rh6G and 450 nm for FITC-dextran. For the programmed release of molecules from microgelsomes, 1 mL aqueous dispersion of microgelsomes encapsulating Rh6G was stirred at 30 °C for different periods (0 – 55 min). The release of Rh6G in the outer solution was measured by recording the absorbance at 530 nm. The kinetics for the release of Rh6G molecules was performed instantly upon the transfer of microgelsomes in the aqueous phase, however, owing to the rapid diffusion of Rh6G, a significant loss of dye was observed during the phase transfer process.

S2.7. Determination of the catalytic activity of microgelsomes

The antioxidant activity of vitamin E encapsulating microgelsomes (En_Vit E) was studied using $ABTS^{\cdot+}$ assay. The assay is based on the reduction of a pre-generated $ABTS^{\cdot+}$ by the antioxidant, vitamin E. For the pre-generation of $ABTS^{\cdot+}$ solution, 7 mM ABTS was mixed with initiator 2.45 mM APS and incubated overnight (12-16 h) in the dark at room temperature. The $ABTS^{\cdot+}$ radical solution was then diluted in ethanol until an absorbance of 0.7 ± 0.03 was reached at 730 nm.[4] Fresh dilution was performed before each assay to ensure the stability of $ABTS^{\cdot+}$ radical solution. For the $ABTS^{\cdot+}$ assay, 10 µL of free vitamin E solution (F_Vit E) (10 % (w/w) or En_Vit E was added to 10 µL diluted $ABTS^{\cdot+}$ radical solution and the total volume was made up to 200 µL using phosphate buffer (pH 7.2). The decrease in absorbance was measured at 730 nm for 6 min (multimode Biotek Synergy H1 microplate reader). The percentage antioxidant activity *(AA)* was calculated using equation (1):[4]

$$AA\ (\%) = \frac{A_S - A_R}{A_S} \times 100 \qquad (1)$$

where A_S is absorbance of diluted $ABTS^{\cdot+}$ at t = 0, A_R is absorbance at t = 6 min.

The catalytic activity of enzyme-encapsulating microgelsomes (prepared via W/O Pickering emulsion) was investigated using HPLC and GC-MS methods. **(Section S1).** For the two-step sequential reaction, 300 μL of laccase-encapsulating microgelsomes (En_lac)/ free laccase (Free lac) was added to acetate buffer (0.1 M, pH 5, for a total volume of 1 mL) containing 1-phenyl ethanol (10 mM) and TEMPO (33 mol%) as chemical mediator. The reaction was stirred for 12 h at 30 °C at 800 rpm. Upon completion, the solution was centrifuged, and the product was collected for the second step of cascade reaction, involving transaminase. To the crude product, TRIS buffer (0.2 M, pH 8, total reaction volume for 2 mL) containing 20 μL alanine (2 M) and 25 μL PLP (40 mM) and 300 μL STH-encapsulating microgelsomes (En_STH/ Free STH) was added. The addition of 0.2 M TRIS buffer (pH 8) increases the pH of the initial reaction mixture from pH 5 to pH 8. The reaction mixture was stirred 12 h at 30 °C at 800 rpm. Upon completion, the reaction mixture was centrifuged and the product obtained was collected for analysis. The formation of acetophenone and S-MBA was quantified by integrating the peaks obtained at a retention time of 5 min and 2.01 min, respectively, measured at an absorbance of 210 nm. The concentration of acetophenone and S-MBA formed during the course of the reaction was calculated using the acetophenone and S-MBA standard plots **(Figure S10(A and B)).**

The one-pot cascade was performed by the co-encapsulation of laccase and STH (LaSh) in microgelsomes. For the enzymatic activity, 300 μL En_LaSh/ Free LaSh (containing 12 mg STH and 5 mg laccase were dispersed in the acetate buffer (0.1 M, pH 5, total reaction volume 2 mL) containing 10 μL of 1-phenyl ethanol (1M), 33 mol% TEMPO, 25 μL PLP (40 mM), and 20 μL alanine (2M). The reaction was shaken at 30 °C, 800 rpm for 12 h. The activity of LaSh was determined by integrating the peak obtained at a retention time of 2.01 min, measured at 210 nm, using the HPLC chromatogram.

One unit of laccase activity was defined as the amount of enzyme required to form 1 mmol acetophenone per minute at 30 °C. BCA assay with a sensitivity of 0.1-1.4 mg/mL was used to determine protein concentration using laccase as the standard protein.

One unit of STH activity was defined as the amount of enzyme required to form 1 mmol S-MBA per minute at 30 °C. Bradford microassay with a sensitivity of 0.2-1mg/mL was used to determine protein concentration using BSA as the standard protein.

S2.8. Labelling of enzymes with fluorescent dyes

To validate the encapsulation of enzymes (transaminase (STH) and laccase (lac)) in microgelsomes, the enzymes, STH and lac were labeled with a fluorescent dye, fluorescein isothiocyanate (FITC), and rhodamine B isothiocyanate (RITC), respectively. Typically, enzymes (5 mg) were dissolved in 2 mL sodium carbonate buffer (pH 8.5, 100 mM), followed by the dropwise addition of 50 μL of fluorescein isothiocyanate (FITC) or rhodamine B isothiocyanate (RITC) dissolved in DMSO solution (1 mg/mL). The solution was stirred at 15 °C for 5 h and purified using an amplicon dialysis tube (10 kDa) via centrifugation, which was then freeze-dried and stored at -20 °C for further use.

S2.9. Labeling microgel particles with fluorescent dye

PNSER microgels (20 mg) were dispersed in 2 mL sodium bicarbonate buffer (pH 8.5, 100 mM). A 50 μL of FITC DMSO solution (1 mg/mL) was added dropwise and the dispersion was stirred at 15 °C for 5 h. The unbound dye molecules were removed by repeated centrifugation at 15000 rpm for 10 min and freeze-dried.

S2.10. Staining oil with fluorescent dye

For the CLSM analysis of microgel-stabilized O/W emulsion, 2-ethyl 1-hexanol was mixed with 5 μL hydrophobic dye Nile Red.

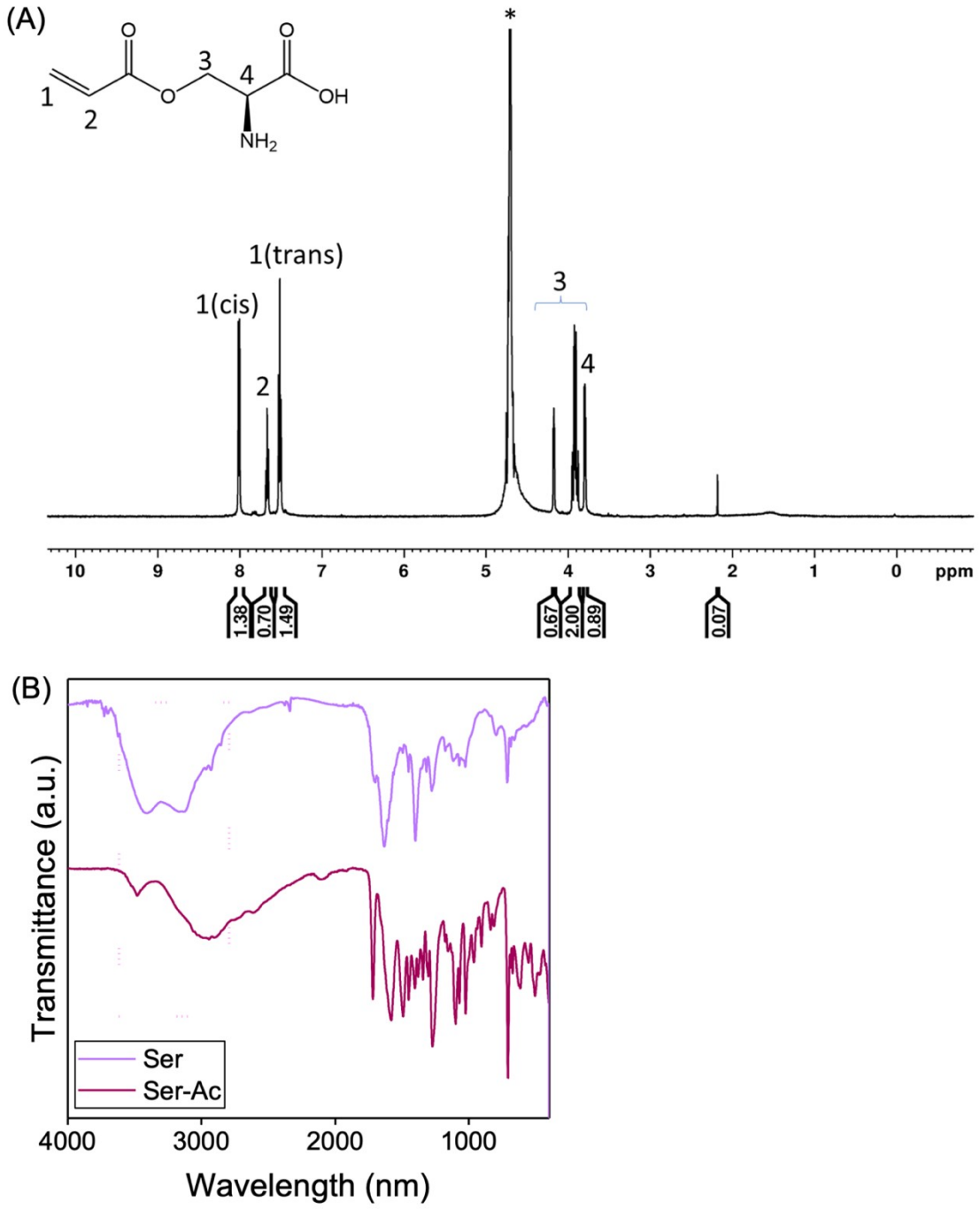

Figure S2. (A) ^{1}H NMR and FTIR spectra of Ser-Ac monomer.

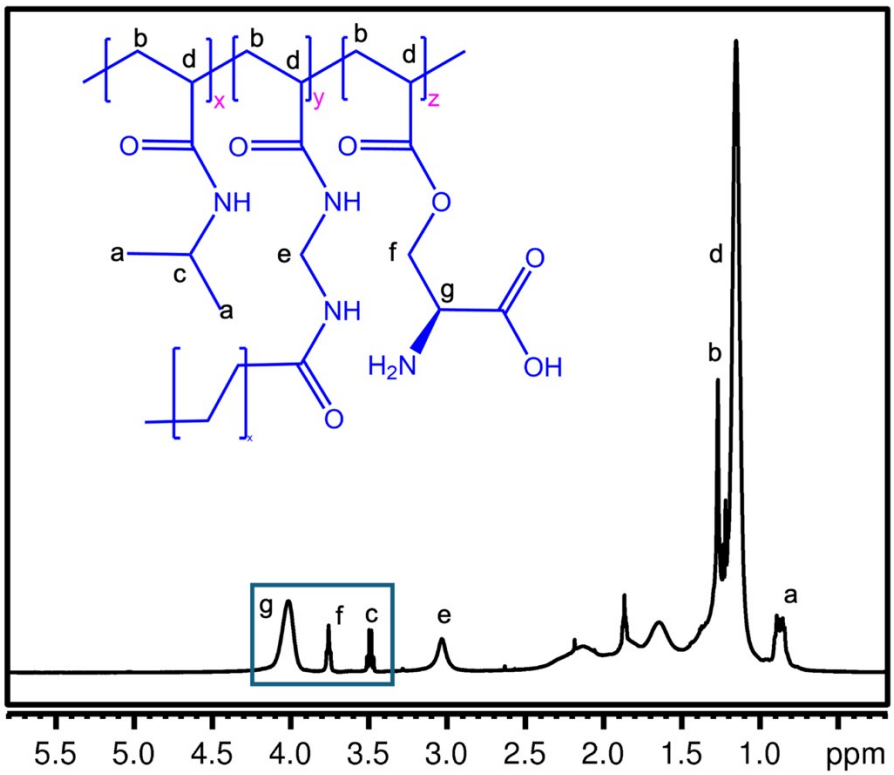

Figure S3. Solid state ^{1}H NMR spectrum of PNSER_20 microgel (400 MHz).

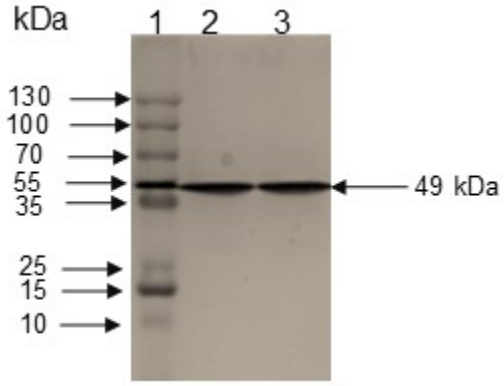

Figure S4. SDS-PAGE gel image for the purification of STH containing lane 1 for molecular weight marker and lanes 2 and 3 for purified protein. Lanes 2 and 3 show a sharp band between 35 – 55 kDa, the expected size of enzyme STH (49 kDa).

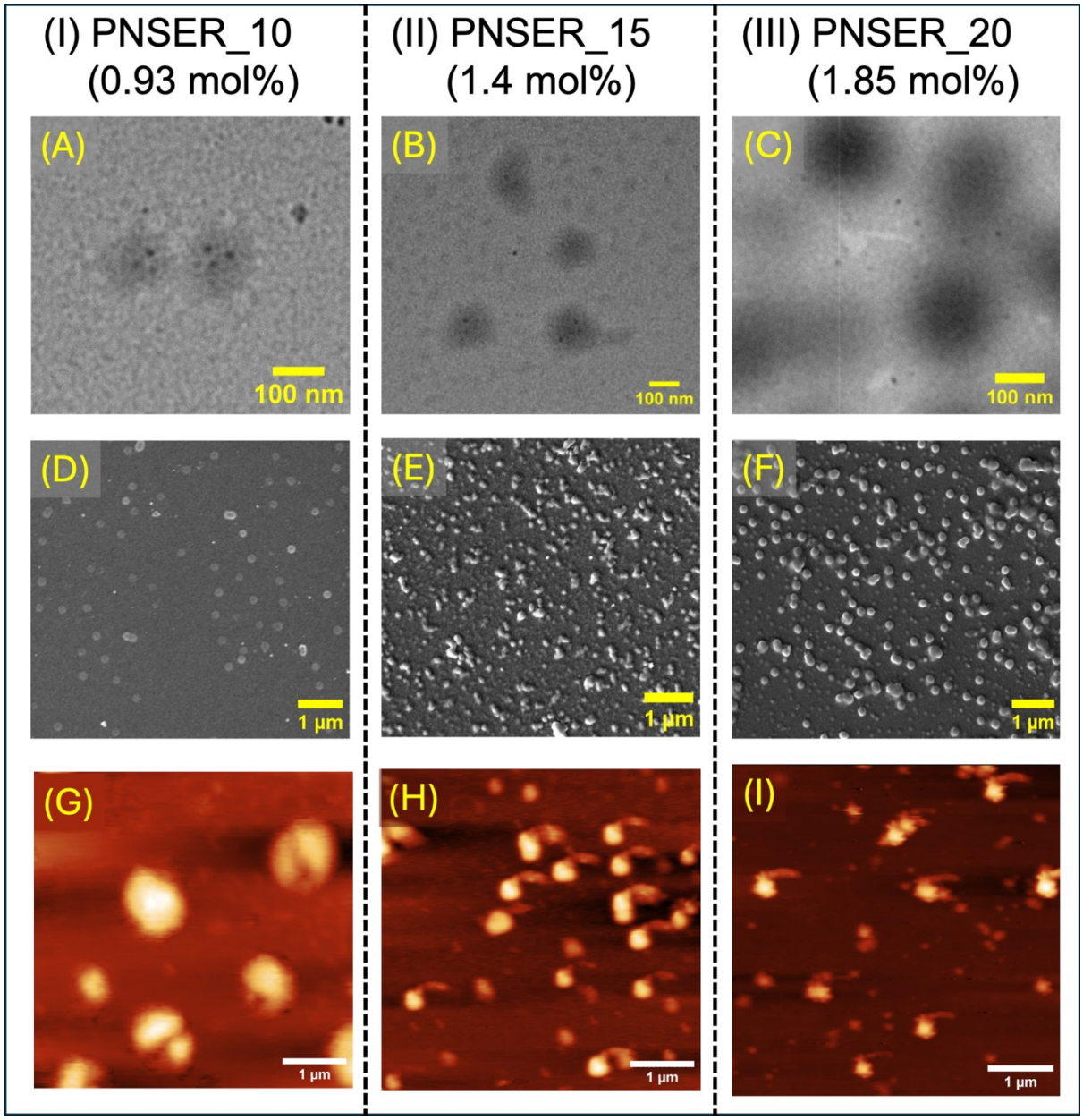

Figure S5. Surface morphology of PNSER_10, PNSER_15, and PNSER_20 microgel particles synthesized using different crosslinker BIS: 0.93 mol%, 1.39 mol%, and 1.85 mol%, respectively.

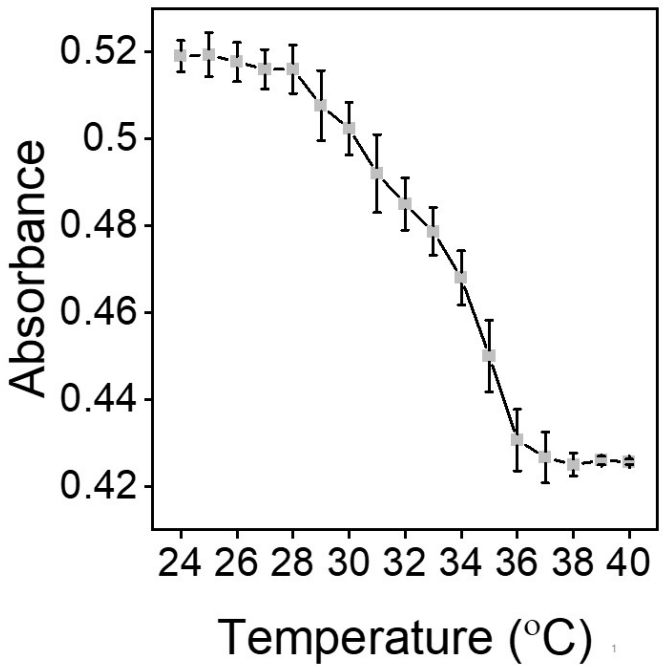

Figure S6. Variation in the size of PNSER_20 microgel as a function of temperature as determined by UV-Vis analysis.

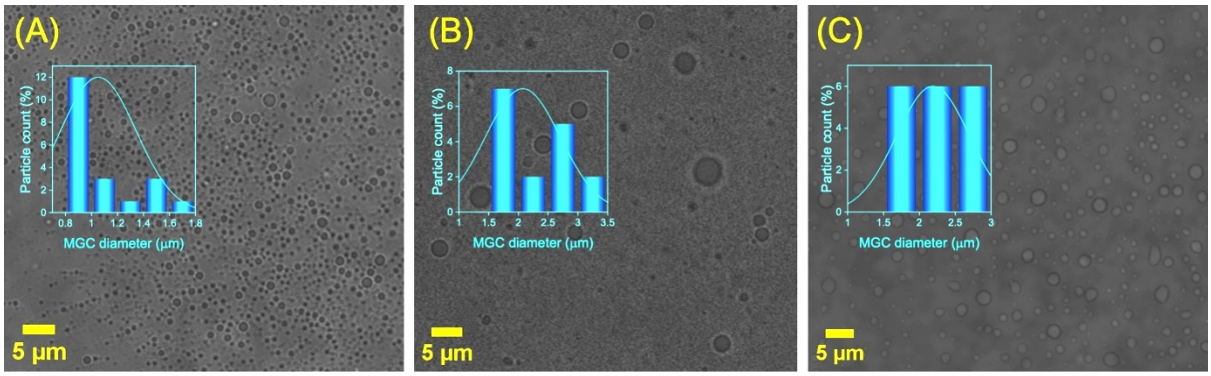

Figure S7. Effect of crosslinker amount of PNSER microgel on the size of microgelsomes. Optical images of microgelsomes prepared using (A) PNSER_10 (0.93 mol%), (B) PNSER_15 (1.39 mol%) and (C) PNSER_20, microgel, (1.85 mol%) particles.

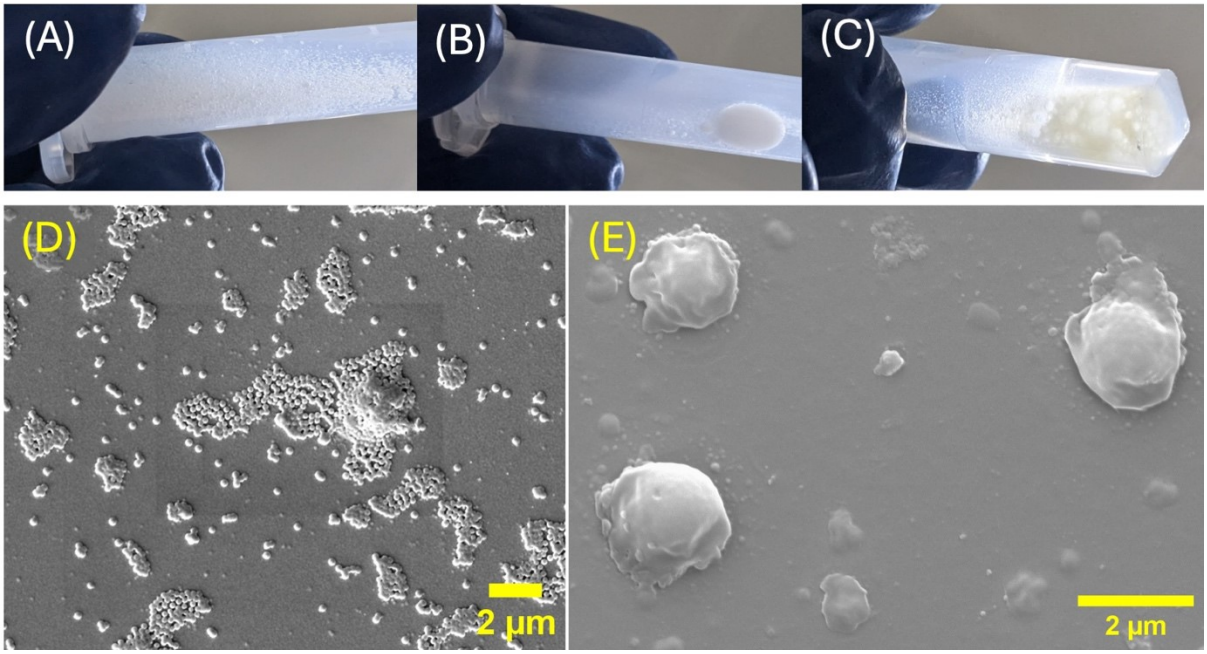

Figure S8. Stability of microgelsomes in different conditions. Images of microgelsomes (A) dispersed in the oil phase, (B) destabilized upon storage below 4 °C, (C) destabilized upon centrifugation. (D) FE-SEM image of disassembled microgelsomes upon storage below 4 °C, (E) FE-SEM image of microgelsomes after one month of storage at room temperature.

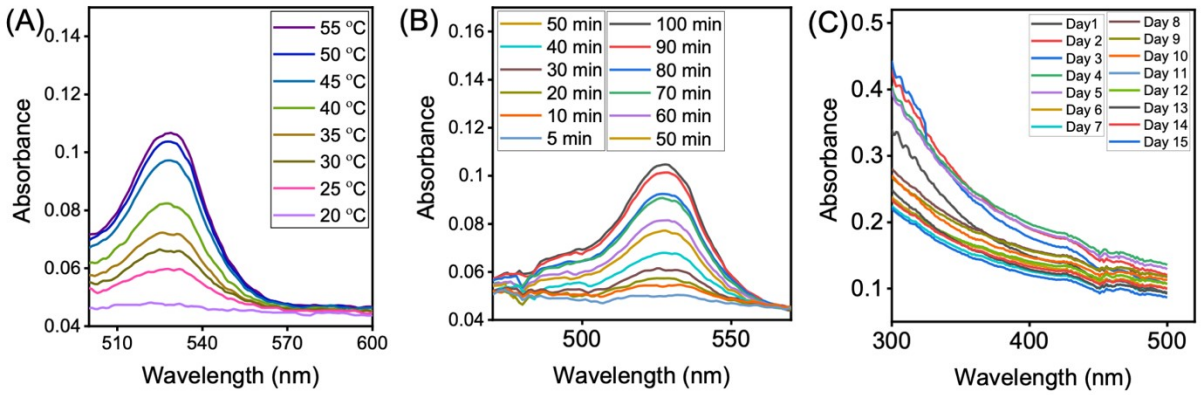

Figure S9. Release profiles of encapsulated Rh6G (A) at different temperatures (20 °C-55 °C) and (B) with time, recorded at 530 nm spectrophotometrically, (C) Release of FITC-dextran for a period of 15 days, absorbance recorded at 450 nm.

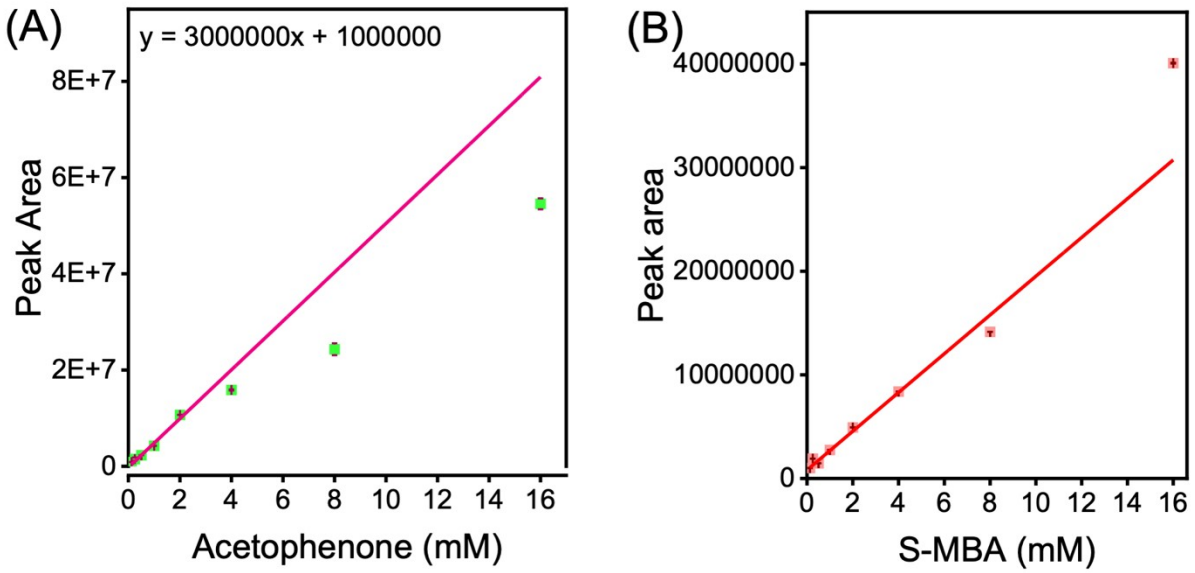

Figure S10. Standard curves for substrates acetophenone and S-MBA.

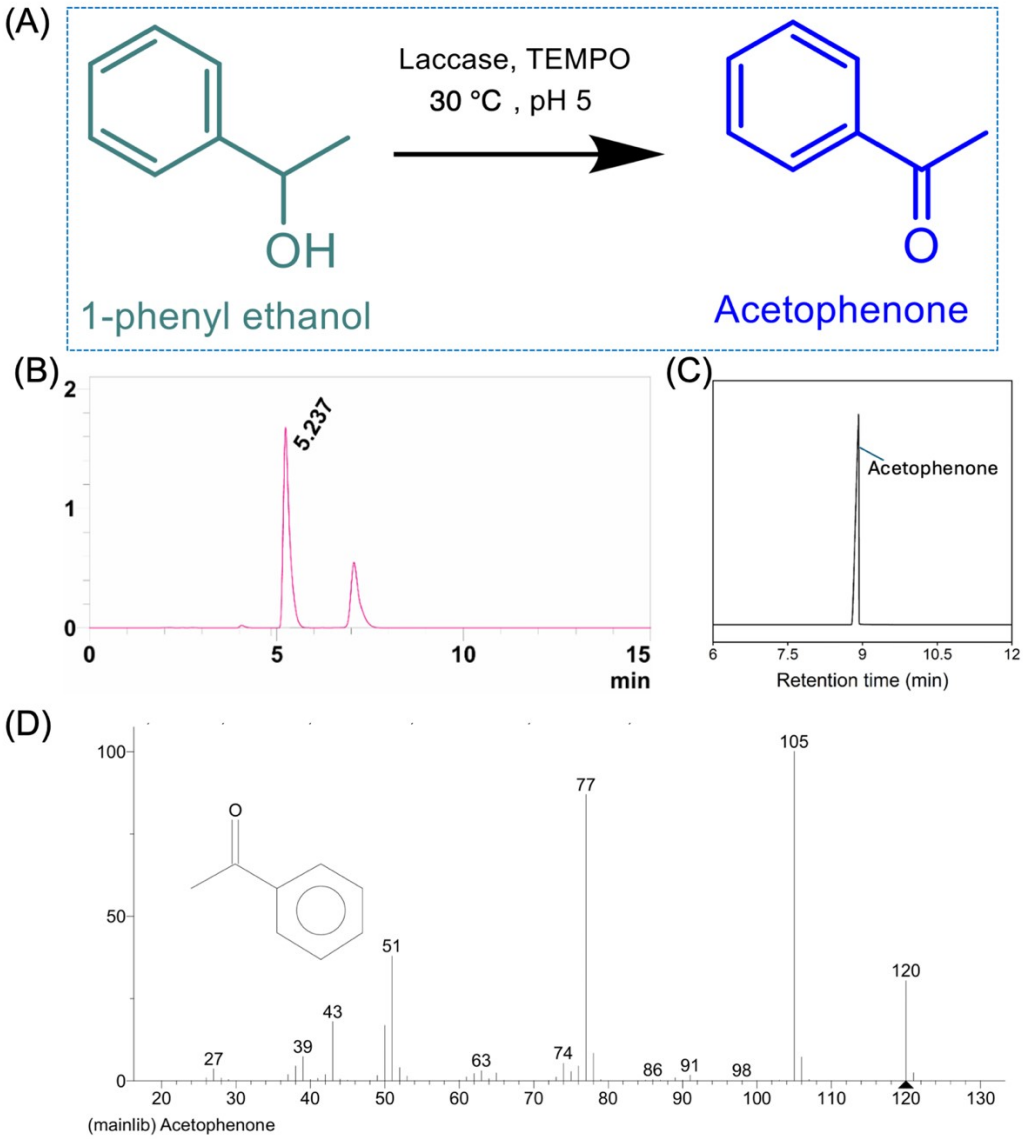

Figure S11. (A) Schematics for the first step of the two-step sequential enzymatic reaction. Laccase-catalysed conversion of 1-phenyl ethanol to acetophenone. Analysis of the reaction mixture obtained after 12 h reaction time using (B) HPLC chromatogram showing peak for acetophenone at retention time 5.2 min, (C) GC-MS chromatogram, (D) mass spectrum for 1-phenyl ethanol, and (E) mass spectrum for acetophenone.

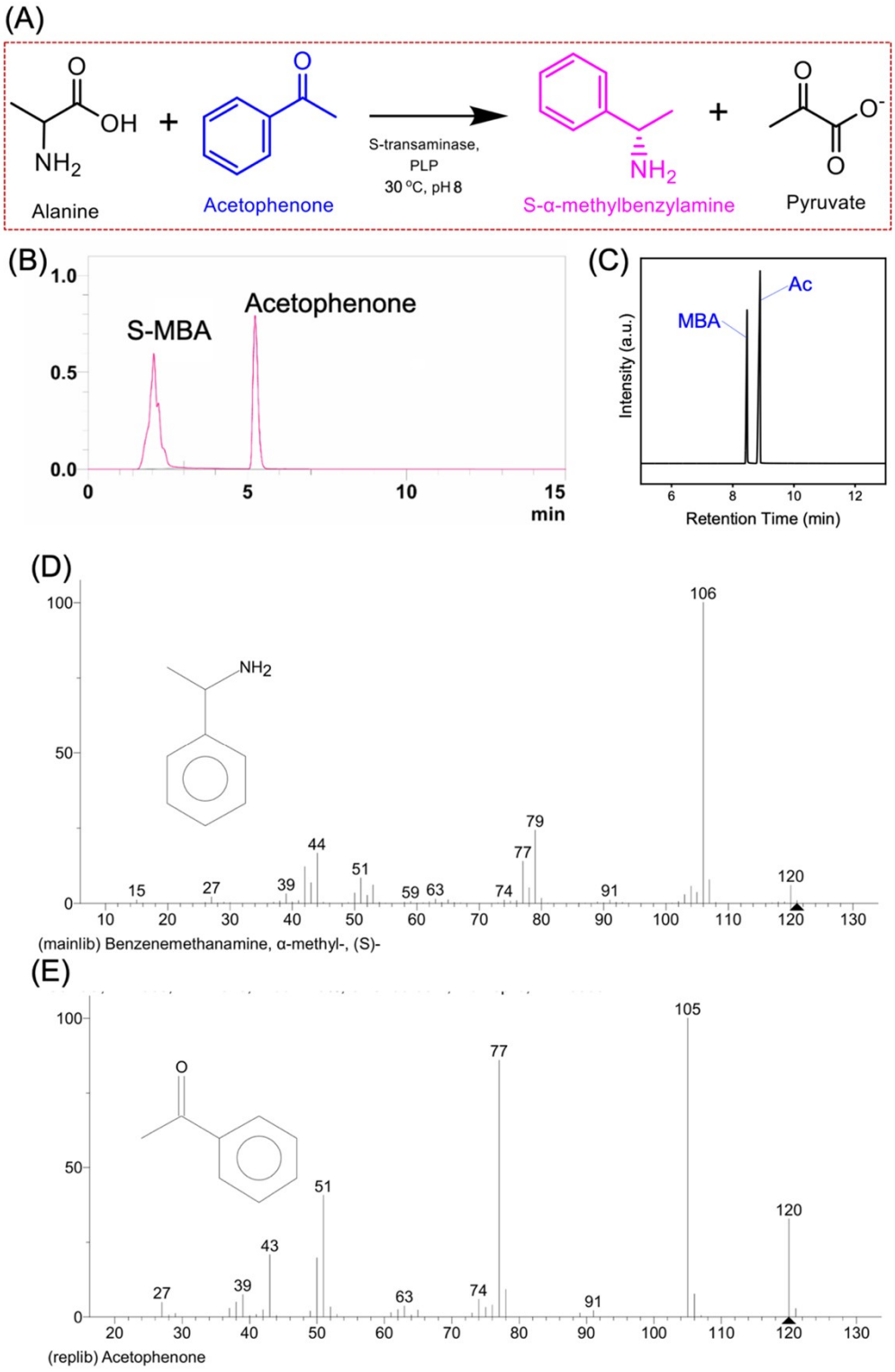

Figure S12. (A) Schematics for the second step of the two-step sequential enzymatic reaction showing transaminase-catalysed conversion of acetophenone to S-methyl benzyl amine (S-MBA). Analysis of the reaction mixture obtained after 12 h reaction time using (B) HPLC chromatogram showing peak for S-MBA at retention time 2.01 min, (C) GC-MS chromatogram, (D) mass spectrum for 1-phenyl ethanol, and (E) mass spectrum for acetophenone.

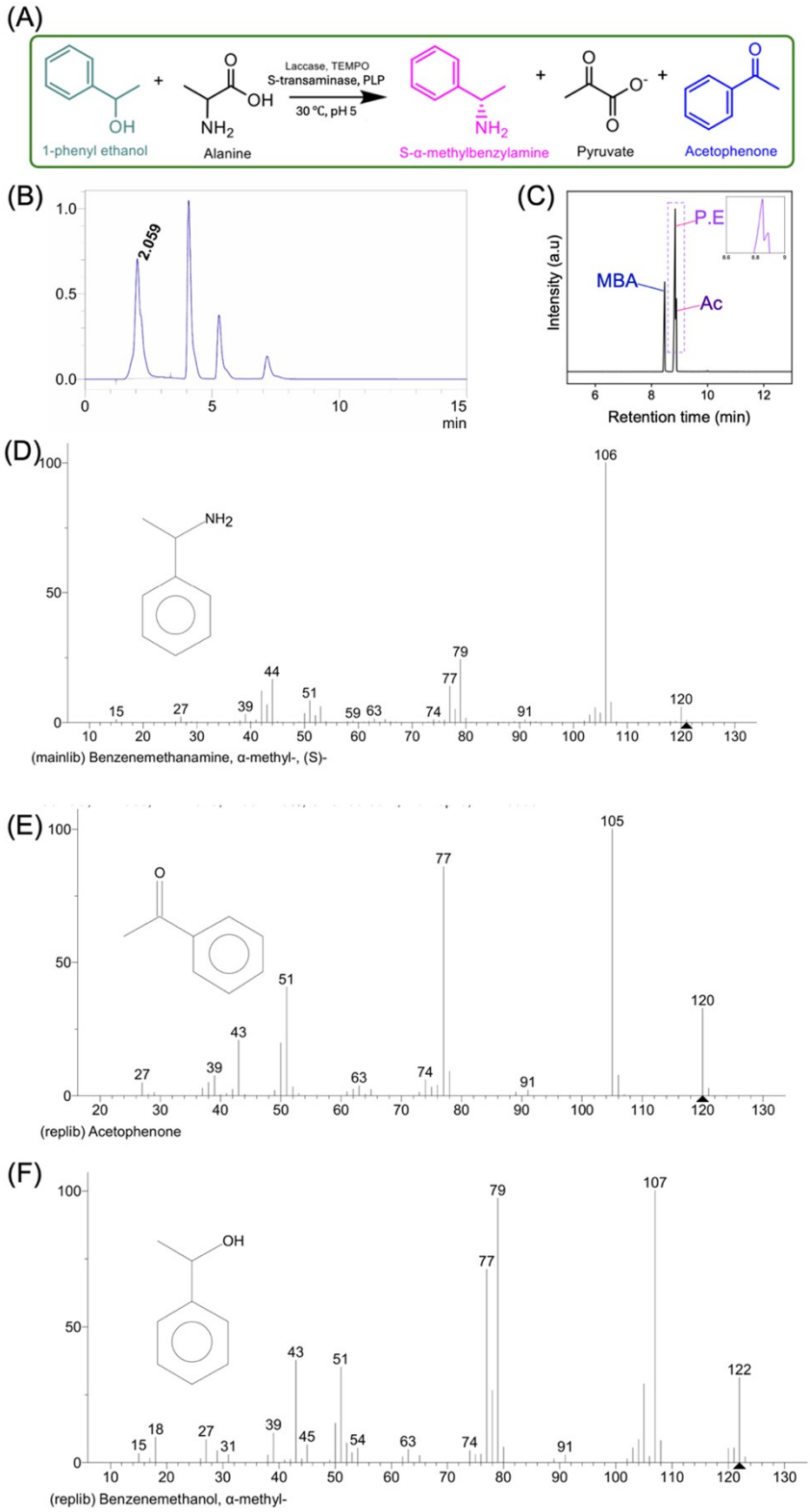

Figure S13. (A) Schematics for the one-pot bienzymatic reaction showing conversion of 1-phenyl ethanol to S-MBA using laccase-transaminase cascade. Analysis of the reaction mixture obtained after 12 h reaction time using (B) HPLC chromatogram showing peak for S-MBA at retention time 2.01 min, (C) GC-MS chromatogram and mass spectrum for (D) S-MBA, (E) acetophenone, and (F) 1-phenyl ethanol.

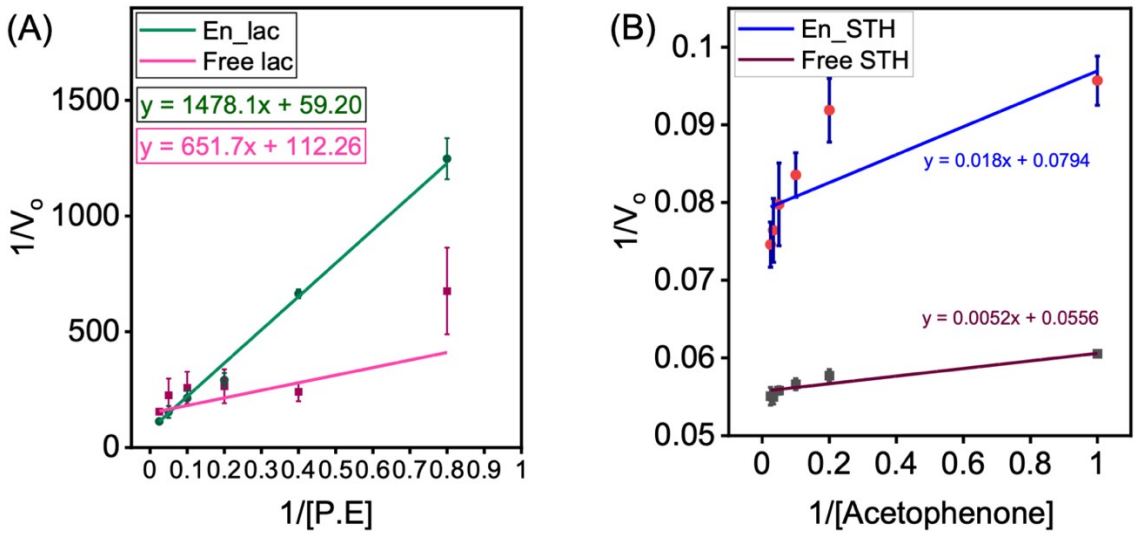

Figure S14. Lineweaver-burk plot for (A) laccase (lac) and (B) transaminase (STH).

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