

Electronic Supporting Information

Substrate-induced assembly of cascade enzymes and catalytic surfactants: nanoarchitectonics at the oil-in-water droplet interface

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1. Materials, Methods and Synthetic protocols:

All commercially available reagents were used as received without any further purification. The chemicals sodium phosphate dibasic (Na_2HPO_4), hexadecyl bromide ($\text{C}_{16}\text{H}_{33}\text{Br}$), Fluorescein isothiocyanate (FITC), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1,2-dichlorobenzene, N, N'-Dicyclohexylcarbodiimide (DCC), 4-Dimethylaminopyridine (DMAP), Glucose, enzyme Horseradish Peroxidase Type 2 (HRP) and Glucose Oxidase from *Aspergillus Niger* (GOx) were procured from Sisco Research Laboratory (SRL), India. 1,9-Dibromononane, potassium thioacetate, di-(2-picolyl)amine (DPA), coumarin-343, Tetramethylrhodamine isothiocyanate were (TRITC), and DMSO were purchased from Sigma-Aldrich. Resorufin was purchased from TCI Chemicals India. Amplex Red was purchased from Invitrogen. The chemicals potassium carbonate (K_2CO_3), and potassium iodide (KI), were also purchased from SRL India, and magnesium nitrate was obtained from Molychem. HPLC-grade methanol and acetonitrile purchased from Sigma-Aldrich were used in the synthesis.

Reactions were monitored by thin layer chromatography (TLC) using silica aluminium sheets with fluorescent indicator at 254 nm (Aldrich). Column Chromatography was performed with 60 - 120 mesh silica gel and neutral alumina. ^1H and ^{13}C NMR spectra were recorded respectively on Bruker 400 MHz and 100 MHz NMR spectrometer using CDCl_3 . Chemical shifts (δ) are given in ppm with TMS as an internal reference. J values are given in Hertz (Hz). High-resolution mass spectra (HRMS) were recorded on Waters QTOF mass spectrometer.

Stock solutions of enzyme/dye were prepared both by weight and by UV-visible spectroscopy using molar extinction ϵ_{280} (HRP) = $102\,000\ \text{M}^{-1}\text{cm}^{-1}$, ϵ_{259} (ATP) = $15400\ \text{M}^{-1}\text{cm}^{-1}$, ϵ_{485} (TRITC) = $62100\ \text{M}^{-1}\text{cm}^{-1}$, ϵ_{495} (FITC) = $68000\ \text{M}^{-1}\text{cm}^{-1}$, ϵ_{280} (GOx) = $220800\ \text{M}^{-1}\text{cm}^{-1}$. UV-vis studies were performed using a Varian Cary 60 (Agilent Technologies) spectrophotometer.

Fluorescence measurements were performed using Cary Eclipse Fluorescence Spectrofluorometer.

All fluorescence microscopy imaging was performed on ZEISS LSM 980 Elyra 7 super-resolution Microscope using a $\times 63$ oil-immersion objective (N.A. 1.4) and a monochrome cooled high-resolution AxioCamMRm Rev. 3 FireWire(D) camera.

The surfactant $C_{16}DPA \bullet Zn^{2+}$ and substrate HPNPP was synthesized and characterized as reported in the literature.^[S1]

HPLC condition: The HPLC analyses were performed using an Agilent 1260 Infinity II HPLC, equipped with a diode array detector (DAD), ($\lambda_{det} = 255$ nm, 290 nm and 315 nm). Conditions: Flow rate: 0.5 ml/min, Isocratic Gradient: 50 % of A - 50 % of B (A: H₂O+0.1% HCOOH, B: ACN+0.1% HCOOH) from 0 to 10min. Column: C18, 4 μ m, 150 \times 4.6mm column. The injection volume was 20 μ l. The temperature of the column was 25°C.

Oil-in-water Droplet formation: For the Oil in water droplet, we used 20 μ l of 1,2-dichlorobenzene in 980 μ l of 5 mM HEPES Buffer pH7 in the presence of 250 μ M $C_{16}DPA \bullet Zn^{2+}$. 2% of 1,2-dichlorobenzene was used during oil in water droplet formation. The mixed solution was vortexed for 60s, which resulted in the formation of micron-sized oil in water droplets.

During the cascade reactions, all the constituents were added along with the surfactant before vortexing, Similarly, during HPNPP catalysis, HPNPP was added before vortexing along with the surfactant and oil.

Preparation of FITC Tagged ALP

FITC-tagged ALP was prepared as given in the literature.^[S2] 1 mg/ml of FITC was dissolved in DMSO, then 100 μ l of this was mixed with 5 mg/ml of ALP (prepared in 0.1M sodium carbonate bicarbonate buffer of pH 9). The solution was kept under the dark at 4 °C for 5 to 6 hrs. After that, the ongoing reaction of tagging was quenched by the addition of 100 μ l of 50 mM of ammonium chloride, and the mixture was kept for more than 2 hrs. Then the reaction mixture was passed through a column made up of Sephadex G-25 and the formed conjugate was purified through the column with 5 mM HEPES buffer (pH= 7). The eluted samples were collected in the different vials and their UV absorption spectra were recorded in which FITC-ALP conjugate showed absorption maxima at 280 nm peak and FITC at 495 nm. Tagging efficiency of ALP was found 0.9, which means a single enzyme has an average of 0.9 FITC molecule.

Preparation of TRITC tagged GOx

TRITC tagged GOx was prepared as given in the literature.^[S2] 1 mg/ml TRITC was dissolved in DMSO, and then 100 μ l of this was mixed with 6 mg/ml of GOx (prepared in 0.1M sodium carbonate bicarbonate buffer of pH 9). The solution was kept under the dark at 4 °C for 5 to 6 hrs. After that, the ongoing reaction of tagging was quenched by the addition of 100 μ l of 50 mM of ammonium chloride, and the mixture was kept for more than 2 hrs. Then the reaction mixture was passed through a column made up of Sephadex G-25 and the formed conjugate was purified through the column with 5 mM HEPES buffer (pH= 7). The eluted samples were collected in the different vials and their UV absorption spectra were recorded in which TRITC-HRP conjugate showed absorption maxima at 280 nm peak and TRITC at 522

nm. Tagging efficiency of GOx was found 2, means a single enzyme has an average of 2 TRITC molecule.

Preparation of TRITC Tagged HRP

TRITC tagged HRP was prepared as given in literature.^[52] 1 mg/ml TRITC was dissolved in DMSO, then 100 μ l of this was mixed with 6 mg/ml of HRP (prepared in 0.1M sodium carbonate bicarbonate buffer of pH 9). The solution was kept under the dark at 4 °C for 5 to 6 hrs. After that, the ongoing reaction of tagging was quenched by the addition of 100 μ l of 50 mM of ammonium chloride and the mixture was kept for more than 2 hrs. Then the reaction mixture was passed through a column made up of Sephadex G-25 and the formed conjugate was purified through the column with 5 mM HEPES buffer (pH= 7). The eluted samples were collected in the different vials and their UV absorption spectra were recorded in which TRITC-HRP conjugate showed absorption maxima at 406 nm peak and TRITC at 522 nm. The tagging efficiency of HRP was found 0.6, means a single enzyme has an average of 0.6 TRITC molecule.

FRET Imaging via Acceptor Photobleaching

Using a 63x oil-immersion objective (N.A 1.4) and a monochrome-cooled high-resolution AxioCamMRm Rev. 3 FireWire(D) camera, metallomicelle enzyme conjugates were captured on camera with a ZEISS LSM 980 Elyra 7 super-resolution microscope. The acceptor in a dual-labeled surfactant stabilized o/w interface was photobleached with a 560 nm laser to measure the FRET efficiency. The rise in donor fluorescence intensity was seen after the

acceptor fluorophore was bleached. Software called Zen Blue 3.2 (ZEISS) was used to estimate FRET efficiency.

The FRET efficiency ($FRET_{eff}$) was measured using the inbuilt software, according to the following equation^[53]:

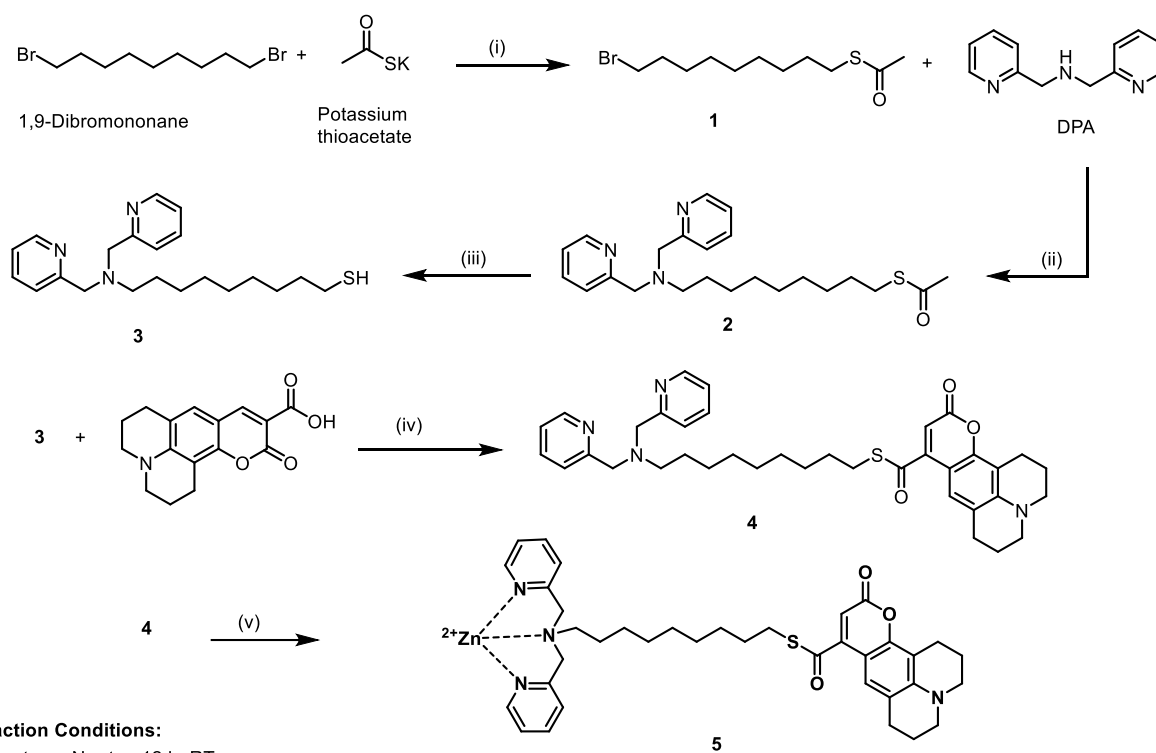
$$FRET_{eff} = [D_{post} - D_{pre}] / D_{post}$$

D_{pre} is the donor fluorescence before photobleaching; D_{post} is the donor fluorescence after photobleaching.

Using ImageJ software, after calibrating the images, the intensity profile data with respect to distance was collected.

Synthetic protocol

Reaction scheme



Reaction Conditions:

- (i) Acetone, N₂ atm, 12 h, RT
- (ii) NaHCO₃, K₂CO₃, ACN, 60 °C, 24 h
- (iii) MeOH, 6 M HCl, 60 °C, 4h
- (iv) DCC, DMAP, dry DCM, 12 h, RT
- (v) Zn(NO₃)₂, MeOH, reflux, 12 h

Synthesis of S-(9-bromononyl) ethanethioate (1)

Compound **1** was synthesized by first dissolving 1,9-dibromononane (1 g, 3.49 mmol) in 15 mL of distilled acetone in a round bottom flask and to this potassium thioacetate (0.39 g, 3.49 mmol) was added. The reaction mixture was allowed to stir at room temperature for 12 h under N₂ atm. The resulting suspension formed was filtered using suction filtration and the filtrate was then evaporated on rotary evaporator to yield crude product as thick oil. The crude was further purified via silica gel (60-120 mesh) column chromatography using hexane as eluent to procure compound **1** as colorless oil with 49% yield.

¹H NMR (400MHz, CDCl₃) δ: 3.42 (t, *J* = 6.8 Hz, 2H), 2.87 (t, *J* = 7.2 Hz, 2H), 2.33 (s, 3H), 1.90-1.83 (m, 2H), 1.61-1.54 (m, 2H), 1.45-1.31 (m, 10H).

¹³C NMR (100 MHz, CDCl₃) δ: 196.0, 77.2, 34.0, 32.7, 30.6, 29.4, 29.2, 29.1, 28.9, 28.7, 28.6, 28.1.

Synthesis of S-(9-(bis(pyridin-2-ylmethyl)amino)nonyl) ethanethioate (2)

Compound **1** (0.3 g, 1.06 mmol) was added to a suspension of dipicolylamine (0.212 g, 1.06 mmol), potassium carbonate (0.441 g, 3.20 mmol) and sodium bicarbonate (0.268 g, 3.20 mmol) in acetonitrile (10 mL). The reaction mixture was stirred at 60 °C for 24 h; after the completion of reaction, the resulting suspension was filtered. The evaporation of the filtrate gave crude product which was further purified via silica gel (60-120 mesh) column chromatography using ethyl acetate/hexane. Yellow oil; yield 40%.

¹H NMR (400 MHz, CDCl₃) δ: 8.50 (d, *J* = 4.3 Hz, 2H), 7.63 (t, *J* = 7.7 Hz, 2H), 7.53 (d, *J* = 7.8 Hz, 2H), 7.12 (t, *J* = 5.8 Hz, 2H), 3.79 (s, 4H), 3.47 (s, MeOH), 2.83 (t, *J* = 7.32 Hz, 2H), 2.51 (t, *J* = 7.2 Hz, 2H), 2.30 (s, 3H), 1.54-1.49 (m, 4H), 1.32-1.20 (m, 10H).

¹³C NMR (100 MHz, CDCl₃) δ: 196.0, 160.1, 148.9, 136.3, 122.8, 121.8, 77.32, 60.5, 54.5, 30.6, 29.4, 29.3, 29.1, 29.0, 28.7.

Synthesis of 9-(Bis(pyridin-2-ylmethyl)amino)nonane-1-thiol (3)

Compound **3** was synthesized by dissolving **2** (0.16 g, 0.40 mmol) in 5 mL MeOH in a 25 mL round bottom flask, and to this 5 mL of 6 M HCl was added. The reaction mixture was heated at 60 °C for 4 h and after evaporation of the solvent under reduced pressure, the aqueous part was neutralized with 2 M NaOH solution. Compound **3** was extracted using diethyl ether which after evaporation gave yellow oil as the pure product with 85% yield.

¹H NMR (400 MHz, CDCl₃) δ: 8.53 (d, *J* = 4.4 Hz, 2H), 7.68-7.64 (m, 2H), 7.55 (d, *J* = 7.8 Hz, 2H), 7.16 (t, *J* = 5.7 Hz, 2H), 3.82 (s, 4H), 2.54-2.47 (m, 4H), 2.08 (CH₃COCH₃), 1.60-1.50 (m, 4H), 1.33-1.21 (m, 10H).

¹³C NMR (100 MHz, CDCl₃) δ: 159.6, 148.5, 136.6, 122.9, 122.0, 77.1, 59.9, 54.4, 33.9, 29.3, 29.2, 28.9, 28.2, 27.1, 26.8, 24.5, 20.8.

Synthesis of *S*-(9-(bis(pyridin-2-ylmethyl)amino)nonyl) 11-oxo-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-pyrano[2,3-*f*]pyrido[3,2-*1-ij*]quinoline-9-carbothioate (4)

To a solution of coumarin-343 (0.023 g, 0.08 mmol), DCC (0.051 g, 0.25 mmol) and DMAP (0.015 g, 0.12 mmol) in dry DCM, compound **3** (0.03 g, 0.08 mmol) was added. The resulting solution was allowed to stir at room temperature for 12 h. The progress of the reaction was monitored by shift in the UV-Vis absorption band from 450 nm to 420 nm. After evaporation of solvent under reduced pressure, the product was purified via column chromatography using neutral alumina. Yellow solid, yield 30%.

¹H NMR (400 MHz, CDCl₃) δ: 8.44 (d, *J* = 4.3 Hz, 2H), 8.14 (d, *J* = 6.2 Hz, 2H), 7.57 (t, *J* = 7.4 Hz, 2H), 7.48-7.46 (m, 2H), 7.06 (t, *J* = 6.1 Hz, 2H), 3.72 (s, 4H), 3.13-3.09 (m, 2H), 2.79-2.75 (m, 2H), 2.44 (t, *J* = 7.2 Hz, 2H), 2.24-2.22 (m, 2H), 1.85-1.83 (m, 4H), 1.67-1.65 (m, 4H), 1.50-1.43 (m, 4H), 1.24-1.18 (m, 10H).

¹³C NMR (100 MHz, CDCl₃) δ: 196.1, 160.1, 154.2, 149.6, 148.9, 139.8, 136.3, 122.8, 121.8, 106.5, 77.2, 60.5, 55.7, 54.4, 39.0, 34.9, 30.6, 29.4, 29.3, 29.1, 29.0, 28.7, 27.2, 27.0, 25.4, 24.7.

HRMS (ESI): *m/z* calcd for C₃₇H₄₄N₄O₃S [M+H]⁺ : 625.3206, found 625.3153.

NMR spectra

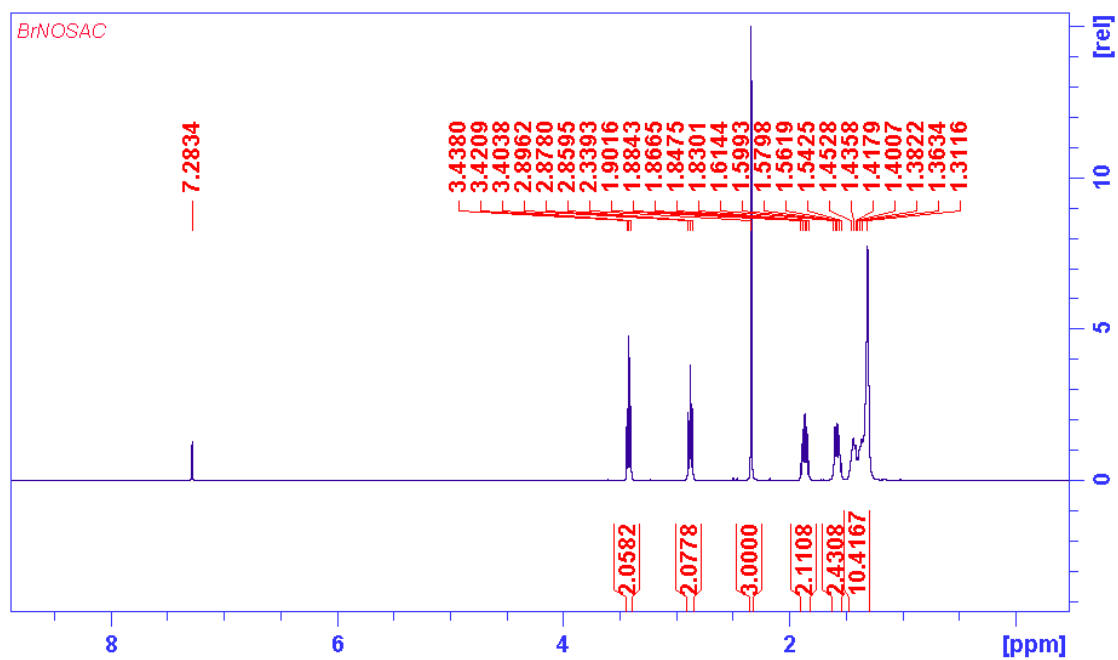


Fig. S1. ^1H NMR of compound **1** in CDCl_3 .

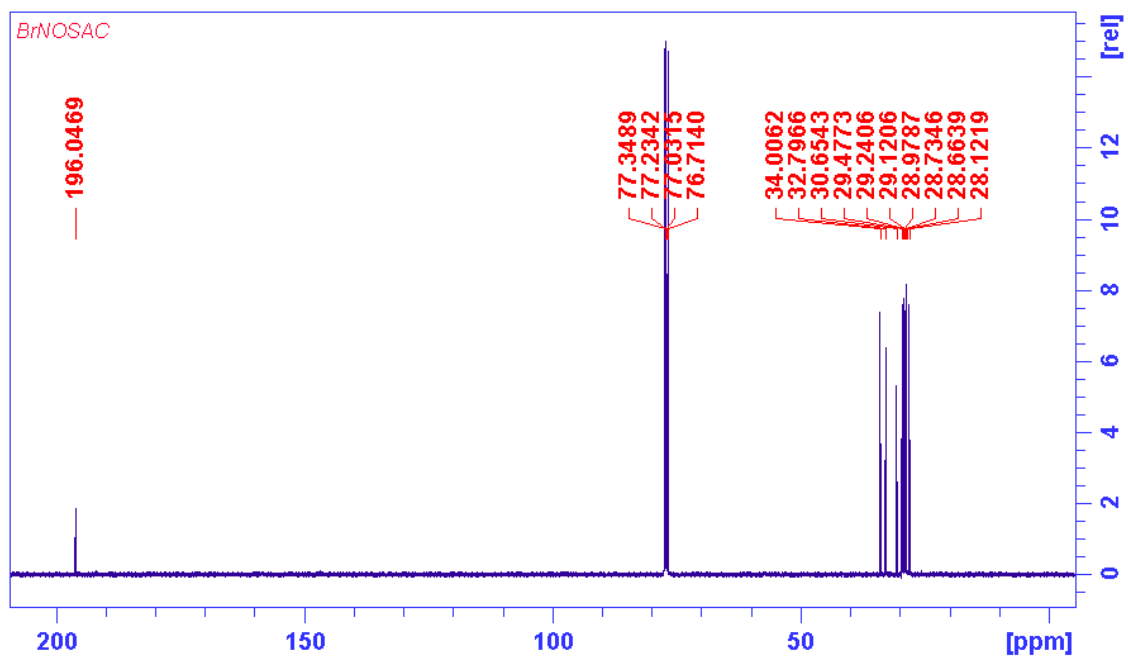


Fig. S2. ^{13}C NMR of compound **1** in CDCl_3 .

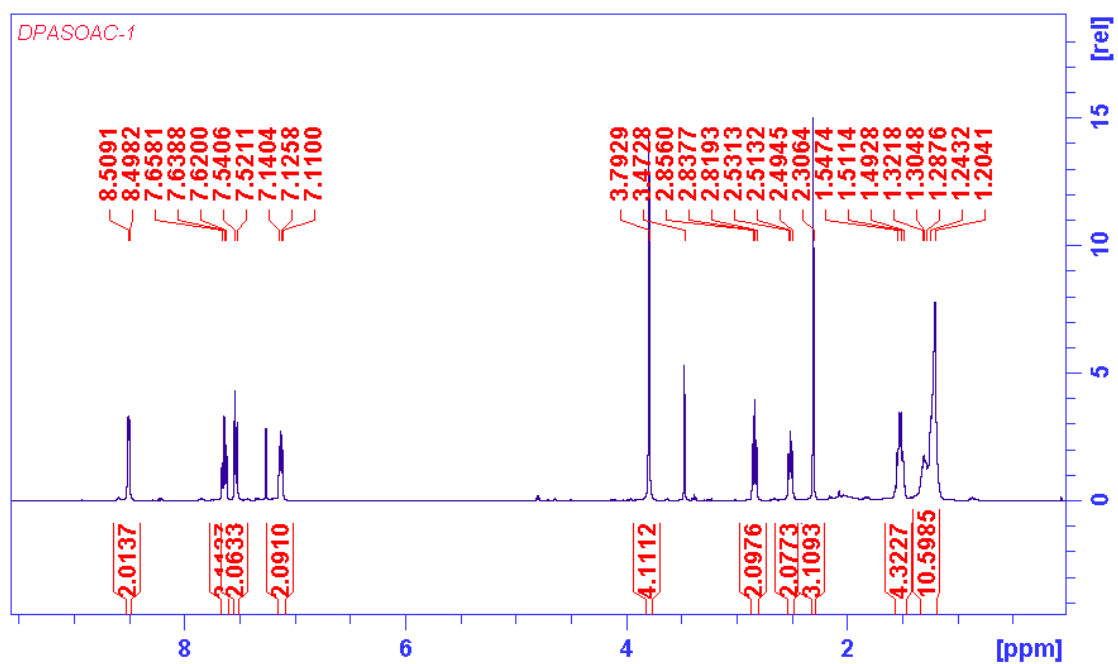


Fig. S3. ^1H NMR of compound **2** in CDCl_3 .

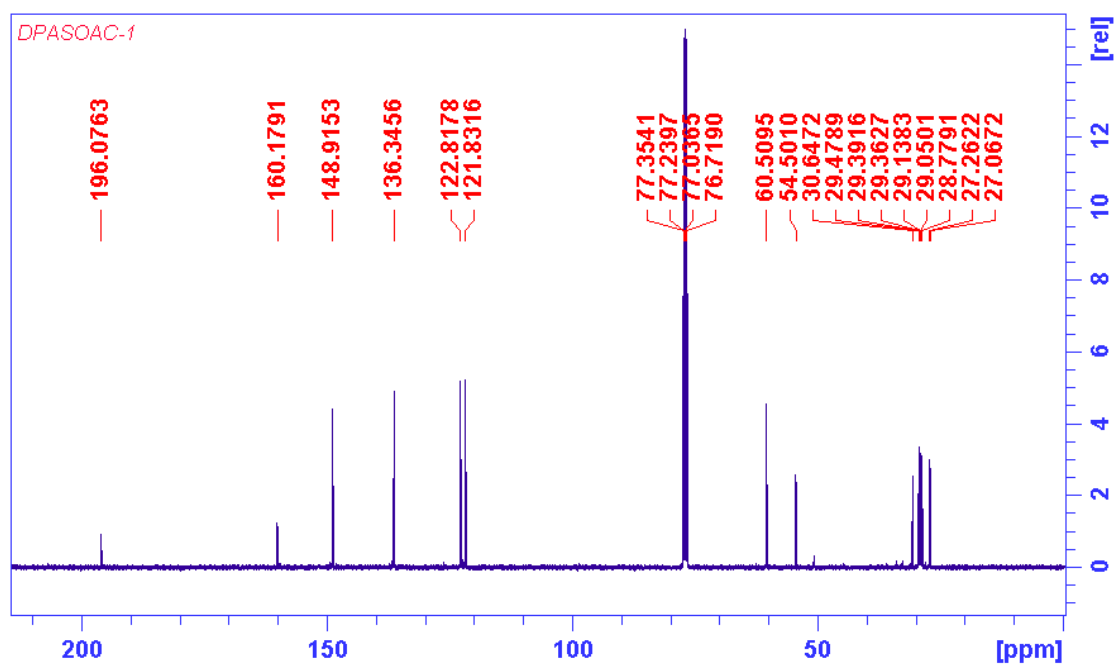


Fig. S4. ^{13}C NMR of compound **2** in CDCl_3 .

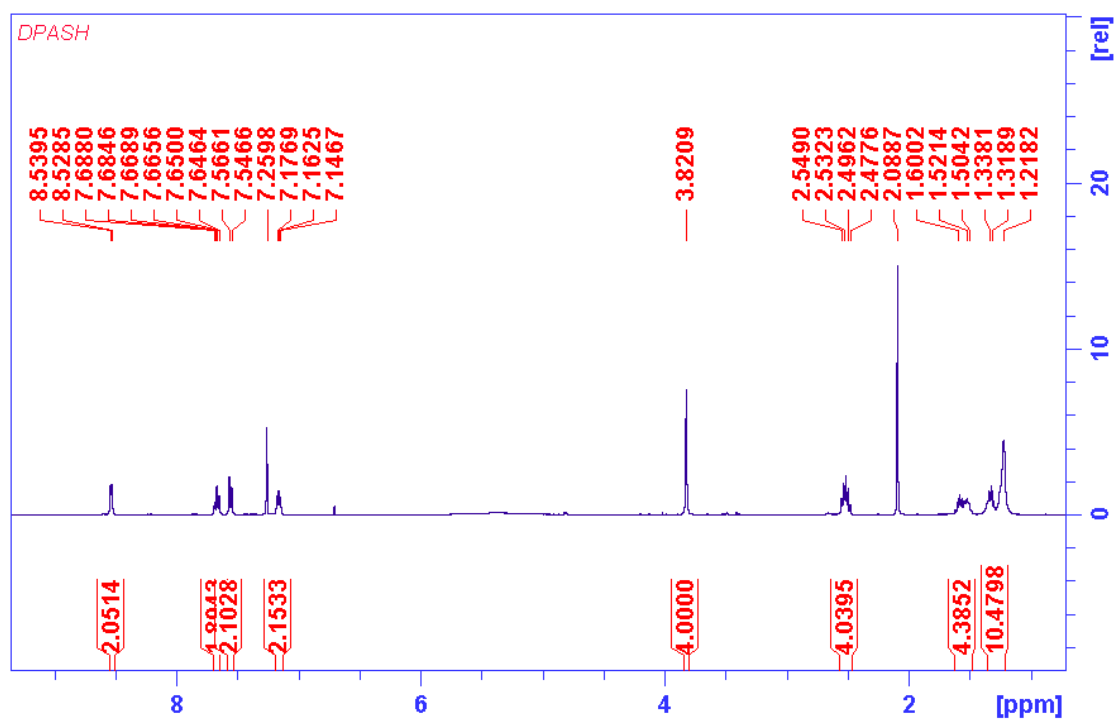


Fig. S5. ^1H NMR of compound **3** in CDCl_3 .

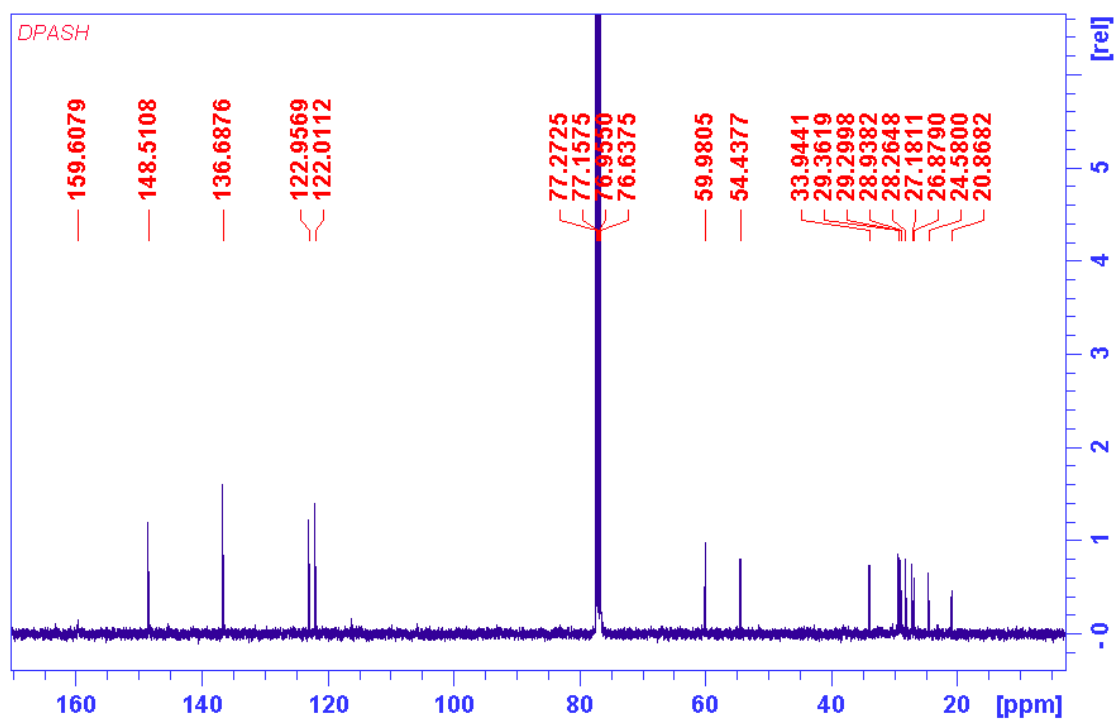


Fig. S6. ^{13}C NMR of compound **3** in CDCl_3 .

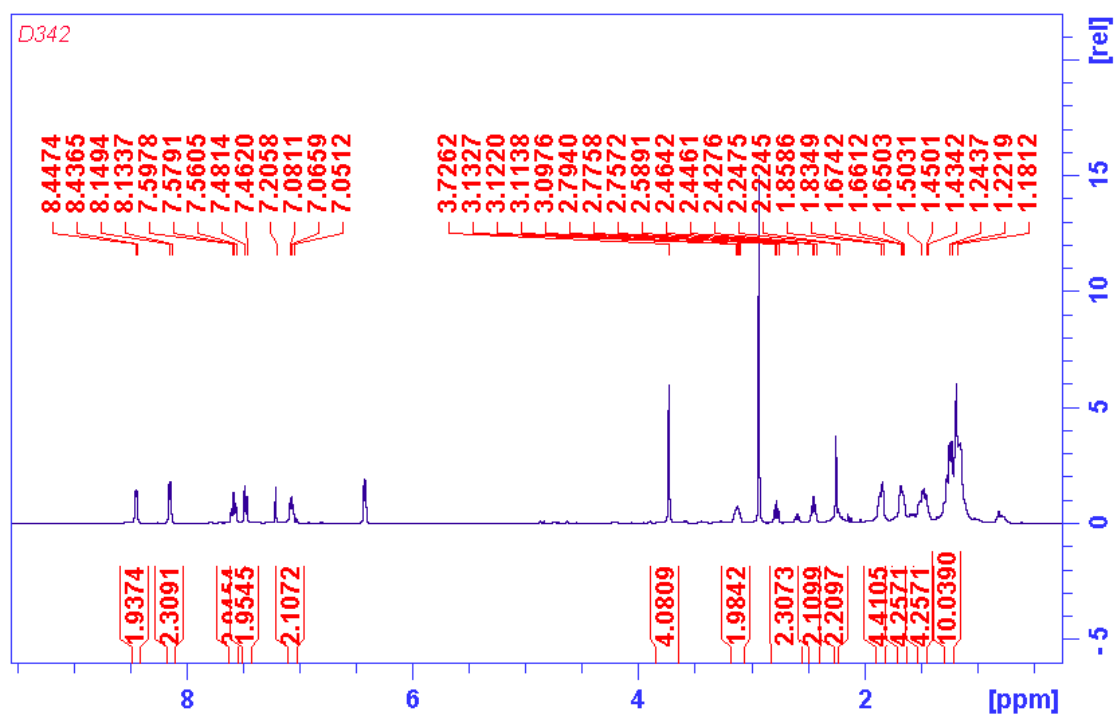


Fig. S7. ^1H NMR of compound **4** in CDCl_3 .

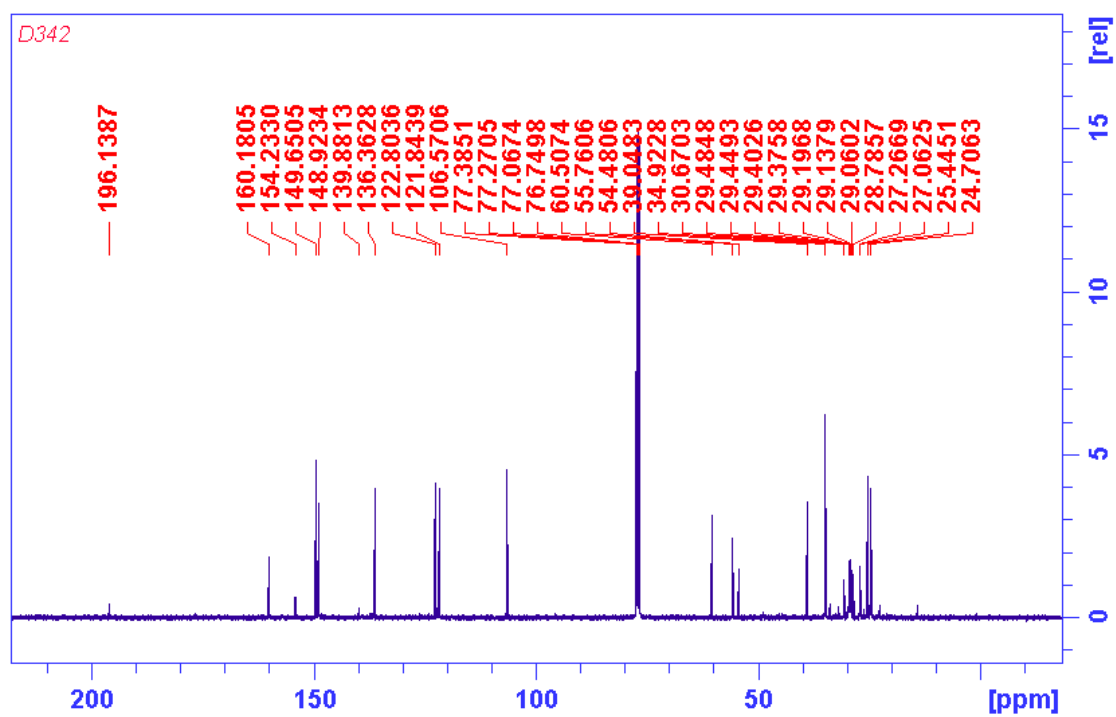


Fig. S8. ^{13}C NMR of compound **4** in CDCl_3 .

Mass Spectra

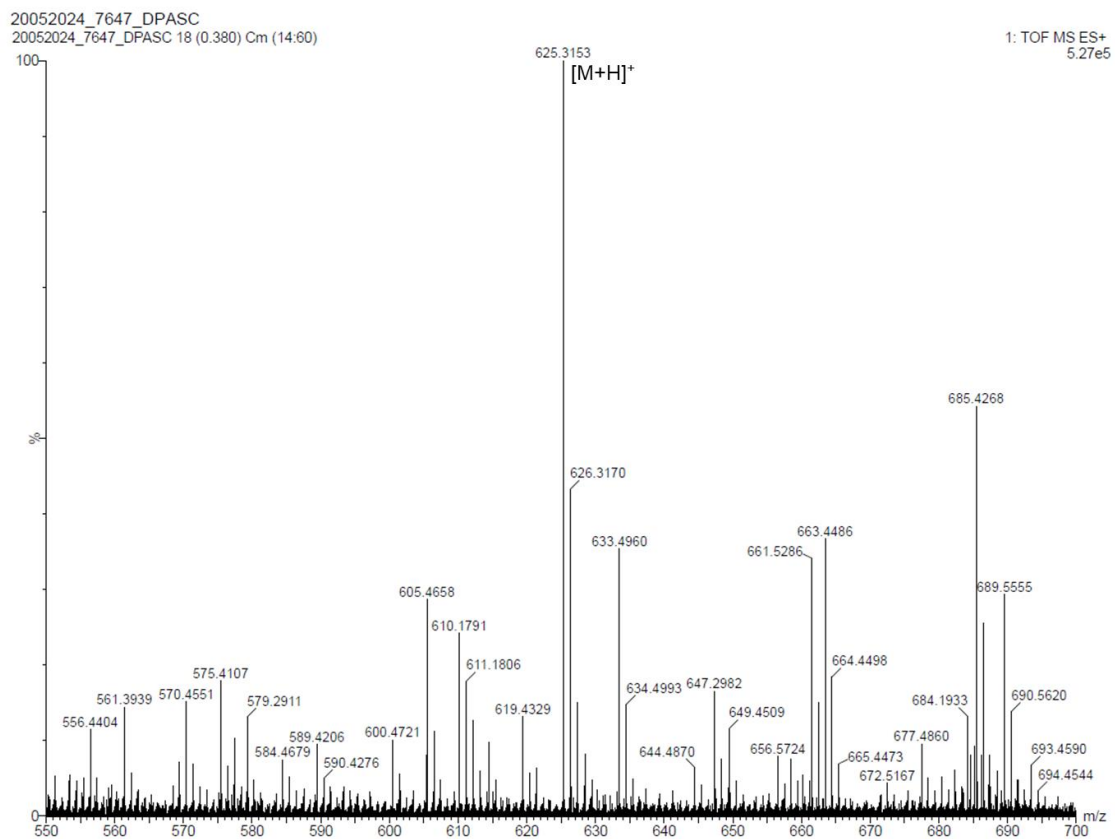


Fig. S9. HRMS of compound **4**, m/z calcd for $C_{37}H_{44}N_4O_3S$ $[M+H]^+$: 625.3206, found 625.3153.

2. Optical Microscopic Images:

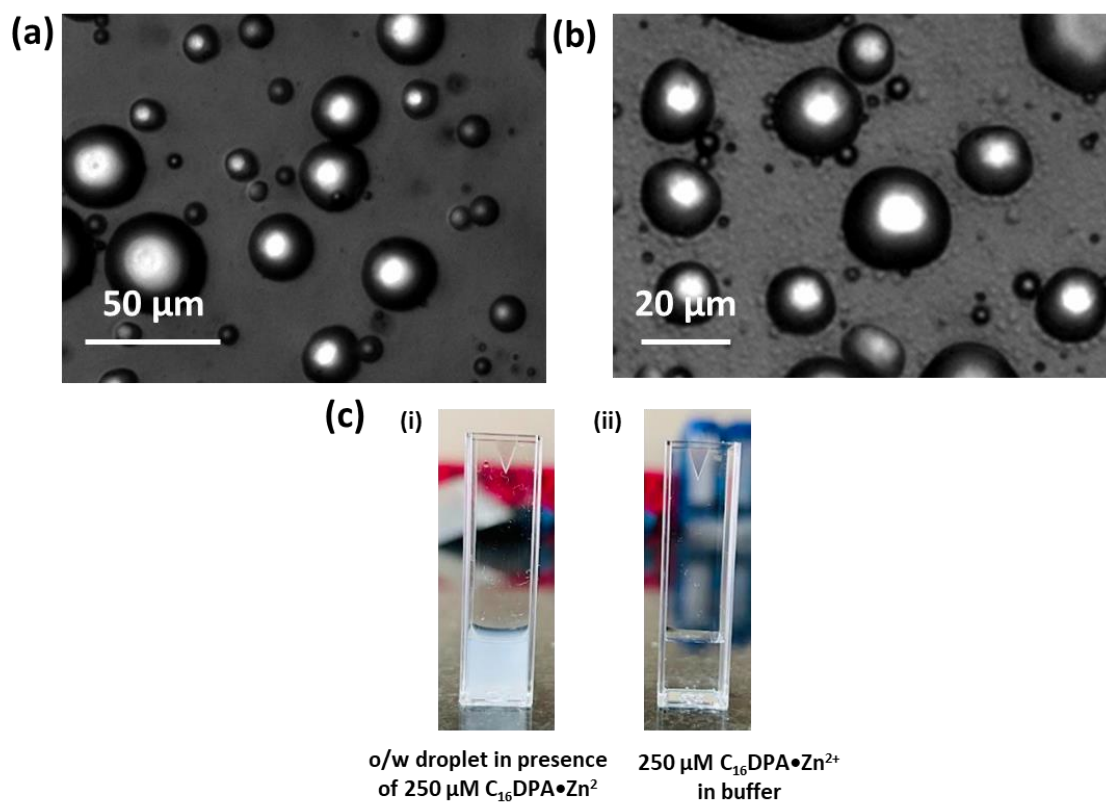


Fig. S10: (a) & (b) Representative Optical Microscopic Image of surfactant stabilized oil – in – water droplet. (c) Representative images of (i) oil in water droplet solution in the presence of 250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ and (ii) only 250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ solution in the buffer. Experimental Condition: $[\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}] = 250 \mu\text{M}$, $[1,2\text{-dichlorobenzene}] = 20 \mu\text{l}$ in 980 μl 5mM HEPES buffer pH7 at 25°C.

3. Enzymatic Cascade Reaction Kinetics:

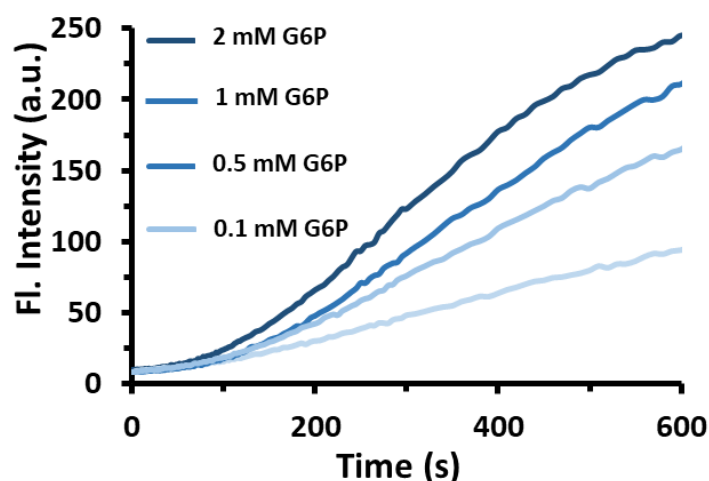


Fig. S11: Representative Fluorescence spectra of the formation of Resorufin over time in the cascade reaction with different conc. of substrate i.e G-6-P. Experimental Condition: [ALP] = 1 μ M, [HRP] = 1 nM, [GOx] = 10 nM, [Amplex Red] = 10 μ M, [Phenylphosphate] = 1mM, [G-6-P] = 0.1 - 2 mM, [C₁₆DPA•Zn²⁺] = 250 μ M, [1,2-dichlorobenzene] = 20 μ l in 980 μ l 5mM HEPES buffer pH7 at 25°C ex/em = 560/585 nm, ex/em slit width = 5/5.

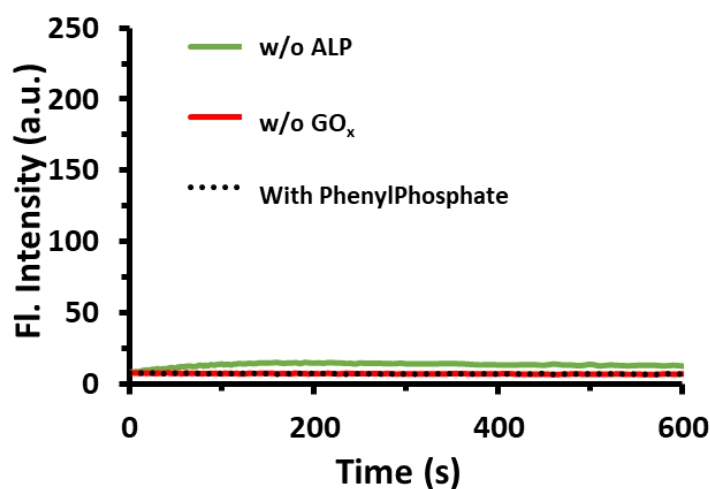


Fig. S12: Representative Fluorescence spectra of the formation of Resorufin over time in the control experiments of cascade reaction without ALP (green line), without GO_x (red line), and with not relevant substrate i.e Phenyl phosphate (dotted black line) of the cascade enzymes. Experimental Condition: [ALP] = 1 μ M, [HRP] = 1 nM, [GOx] = 10 nM, [Amplex Red] = 10 μ M, [Phenylphosphate] = 1mM, [G-6-P] = 1 mM, [C₁₆DPA•Zn²⁺] = 250 μ M, [1,2-dichlorobenzene] = 20 μ l in 980 μ l 5mM HEPES buffer pH7 at 25°C ex/em = 560/585 nm, ex/em slit width = 5/5.

4. Binding of Enzymes on surfactant stabilized o/w interface :

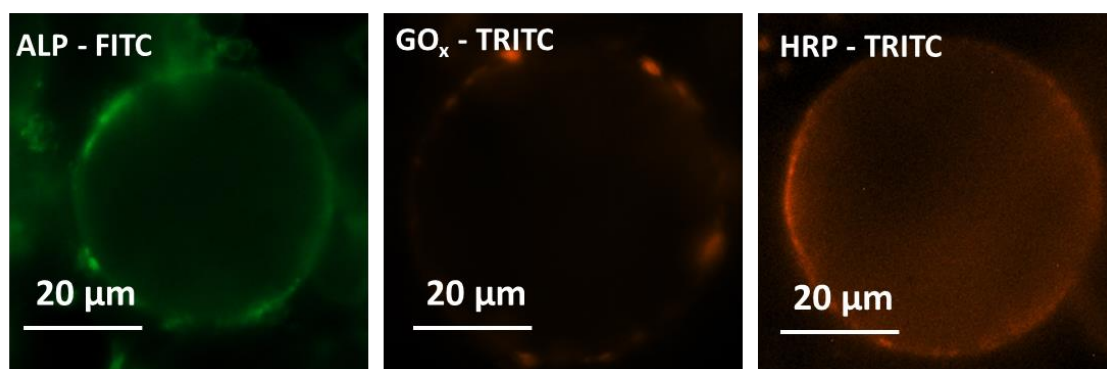


Fig. S13: Fluorescent microscopic images to show the binding of the fluorescently labeled enzymes, (ALP, GOx and HRP) used in this study at the interface. Here FITC and TRITC represent for fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate, respectively. Experimental Condition: [ALP-FITC] = 1 μM , [HRP-FITC] = 0.5 μM , [GOx-FITC] = 0.5 μM [$\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$] = 250 μM , [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

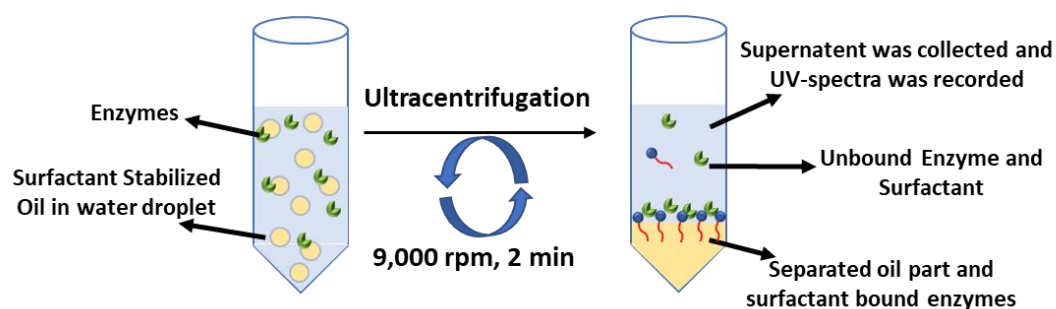


Fig. S14: Schematic representation for determination of bound enzymes at the o/w interface.

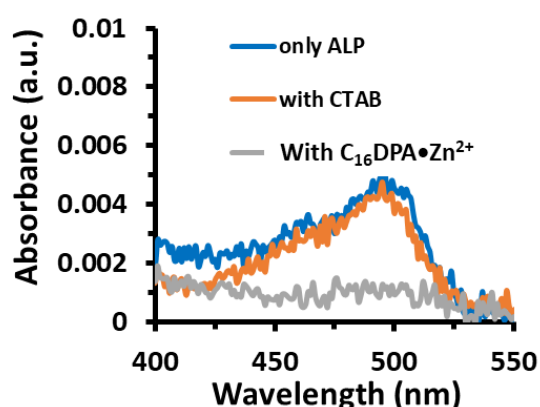


Fig. S15: Representative UV Spectra of the aqueous layer which represent unbound enzyme ALP- FITC after centrifugation of o/w droplet in the presence and absence of surfactants CTAB and $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$, in the system. Centrifugation was done at 9000 rpm for 2 minutes. Experimental Condition: [ALP-FITC] = 1 μM , [CTAB] = 250 μM , [$\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$] = 250 μM , [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

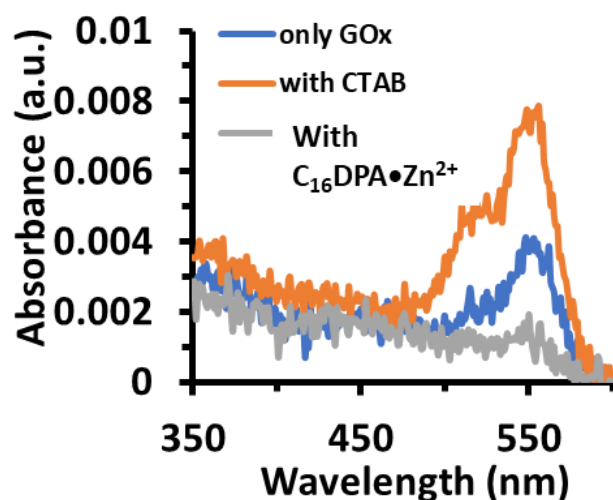


Fig. S16: Representative UV Spectra of the aqueous layer which represent unbound enzyme GOx - TRITC after centrifugation of o/w droplet in the presence and absence of surfactants CTAB and C₁₆DPA•Zn²⁺, in the system. Centrifugation was done at 9000 rpm for 2 minutes. Experimental Condition: [GOx-TRITC] = 1 μM, [CTAB] = 250 μM, [C₁₆DPA•Zn²⁺] = 250 μM, [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

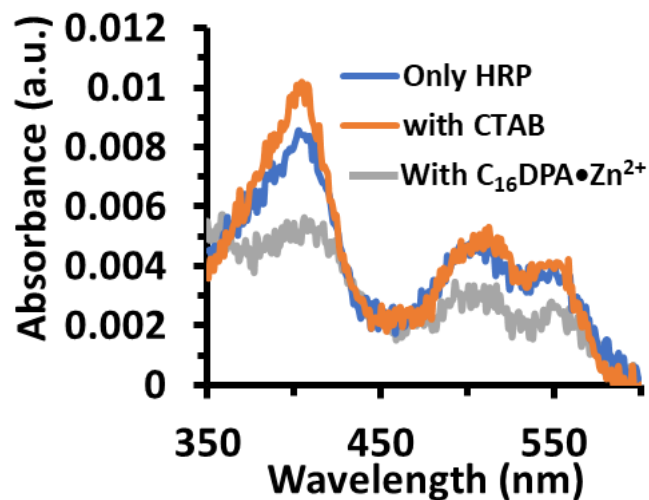


Fig. S17: Representative UV Spectra of the aqueous layer which represent unbound enzyme HRP- TRITC)after centrifugation of o/w droplet in the presence and absence of surfactants CTAB and C₁₆DPA•Zn²⁺, in the system. Centrifugation was done at 9000 rpm for 2 minutes. Experimental Condition: [HRP-TRITC] = 1 μM, [CTAB] = 250 μM, [C₁₆DPA•Zn²⁺] = 250 μM, [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

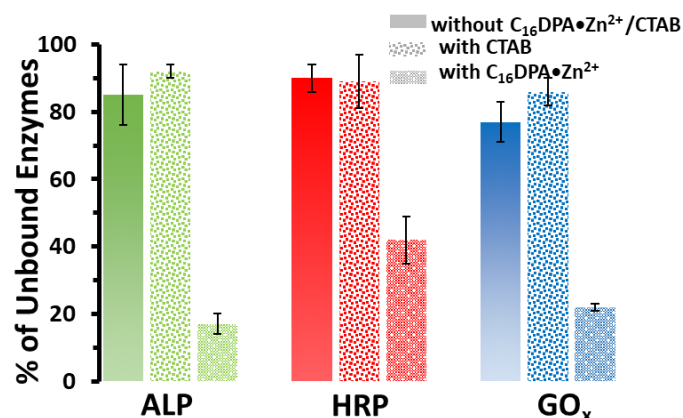


Fig. S18: Representative graph showing percentage of unbound enzymes which represent the amount of enzyme found in the aqueous layer after centrifugation of o/w droplet in the presence of CTAB, $C_{16}DPA \bullet Zn^{2+}$ in the system. Error bars are the standard deviation of three independent experiments. Enzyme used 1 μ M in each case. Experimental Condition: [ALP-FITC] = 1 μ M, [GOx-TRITC] = 1 μ M, [HRP-TRITC] = 1 μ M, [CTAB] = 250 μ M, [$C_{16}DPA \bullet Zn^{2+}$] = 250 μ M, [1,2-dichlorobenzene] = 20 μ l in 980 μ l 5mM HEPES buffer pH7 at 25°C.

This experiment revealed interfacial binding ability of ALP and GOx is around 80%, whereas for HRP it is close to 60%, in our experimental conditions.

5. FRET Efficiency:

(a) FRET Efficiency via Acceptor Photobleaching:

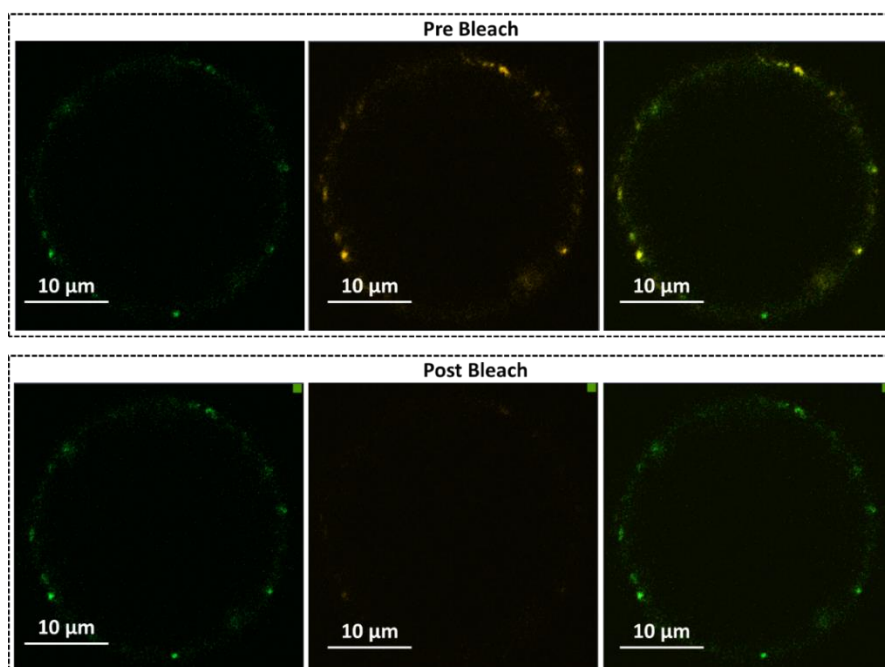


Fig. S19: Representative microscopic images of the surfactant stabilized o/w interface with FITC-ALP, TRITC-GOx and untagged HRP before and after photobleaching of TRITC. Experimental Condition : [ALP-FITC] = 1 μ M, [GOx-TRITC] = 0.5 μ M, [HRP] = 0.5 μ M, [$C_{16}DPA \bullet Zn^{2+}$] = 250 μ M, [1,2-dichlorobenzene] = 20 μ l in 980 μ l 5mM HEPES buffer pH7 at 25°C.

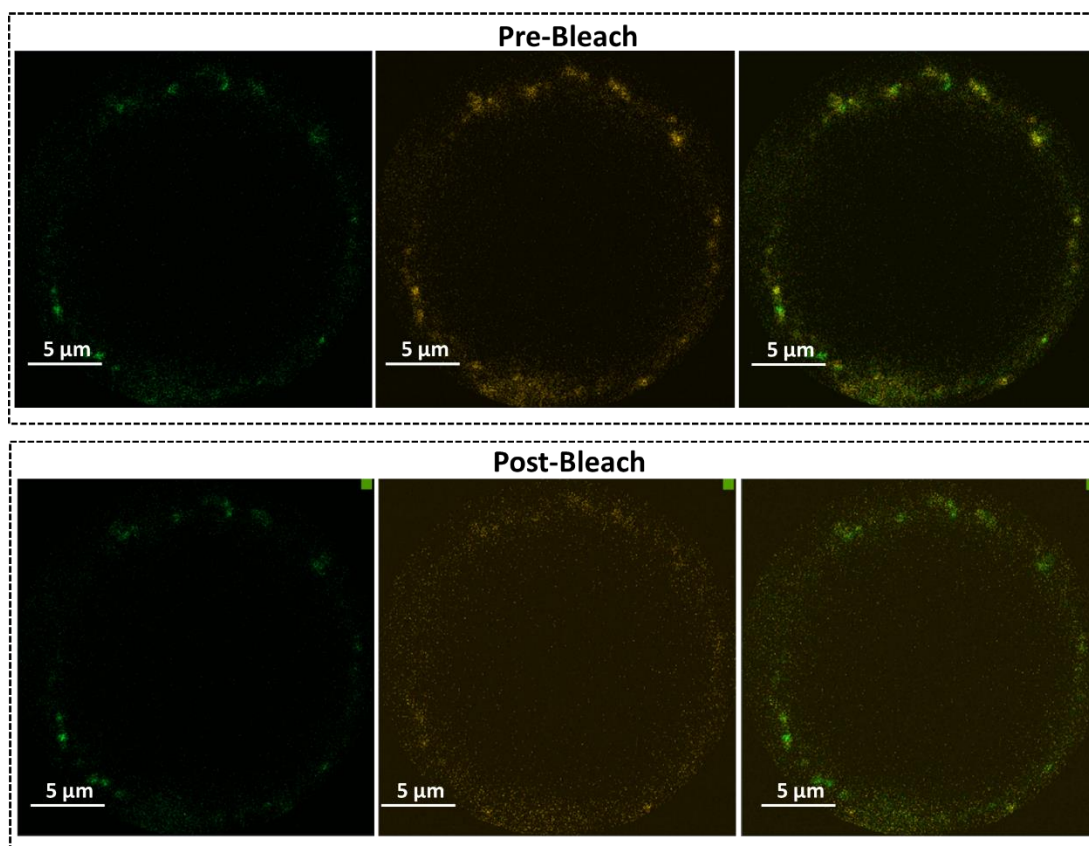


Fig. S20: Representative microscopic images of the surfactant stabilized o/w interface with FITC-ALP, TRITC-GOx and untagged HRP in the presence of Glucose + Phosphate before and after photobleaching of TRITC. Experimental Condition: [ALP-FITC] = 1 μM, [GOx-TRITC] = 0.5 μM, [HRP] = 0.5 μM, [C₁₆DPA•Zn²⁺] = 250 μM, [Glucose] = 1 mM, [Na₂HPO₄] = 1 mM, [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

(b) FRET Efficiency via Steady State Spectroscopy:

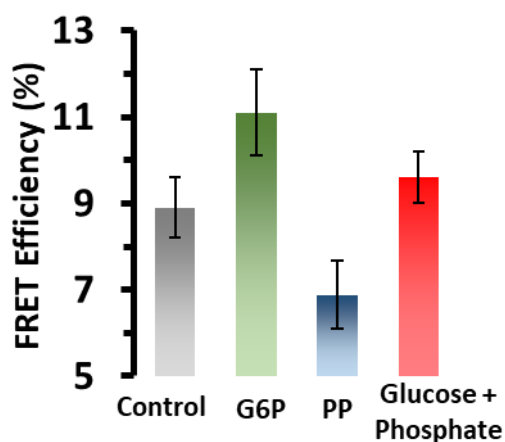


Fig. S21: FRET efficiency calculated from steady state fluorescence in the absence and presence of G-6-P, Glu + Pi and PP. Here we have used FITC tagged ALP, TRITC Tagged GOx, and untagged HRP during the FRET. Experimental condition: [ALP-FITC] = 1 μ M, [GOx-TRITC] = 0.5 μ M, [HRP] = 0.5 μ M, [C₁₆DPA•Zn²⁺] = 250 μ M, [G-6-P] = 1 mM, [Phenyl Phosphate] = 1mM, [Glucose] = 1 mM, [Na₂HPO₄] = 1 mM, [1,2-dichlorobenzene] = 20 μ l in 980 μ l 5mM HEPES buffer pH7 at 25°C.

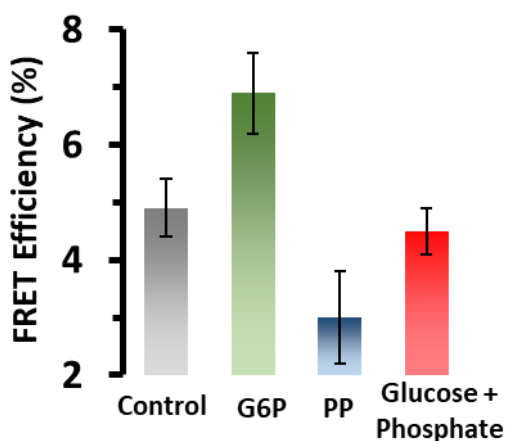


Fig. S22: FRET efficiency calculated from steady state fluorescence in the absence and presence of G-6-P, Glu + Pi and PP. Here we have used FITC tagged ALP, TRITC Tagged HRP, and untagged GOx during the FRET. Experimental condition: [ALP-FITC] = 1 μ M, [HRP-TRITC] = 0.5 μ M, [GOx] = 0.5 μ M, [C₁₆DPA•Zn²⁺] = 250 μ M, [G-6-P] = 1 mM, [Phenyl Phosphate] = 1mM, [Glucose] = 1 mM, [Na₂HPO₄] = 1 mM, [1,2-dichlorobenzene] = 20 μ l in 980 μ l 5mM HEPES buffer pH7 at 25°C.

6. Two color confocal Microscopic Images:

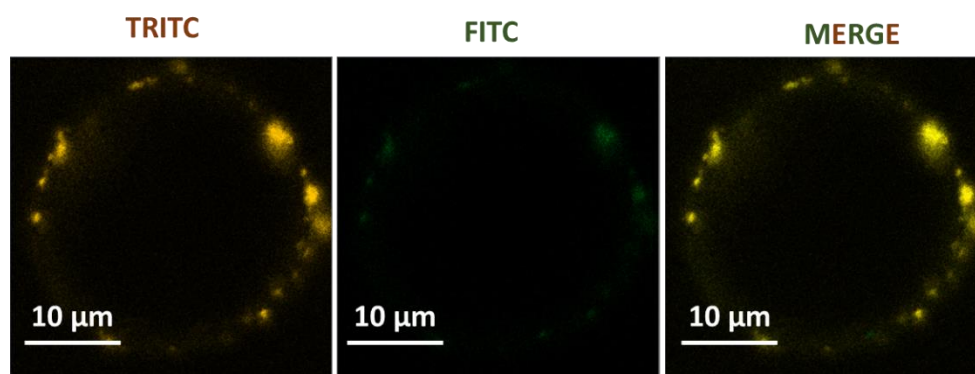


Fig. S23: Representative Two color images of surfactant stabilized o/w interface with GOx-TRITC, ALP-FITC and untagged HRP in the absence of substrate. Experimental condition: [ALP-FITC] = 1 μM , [GOx-TRITC] = 0.5 μM , [HRP] = 0.5 μM , [$\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$] = 250 μM , [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

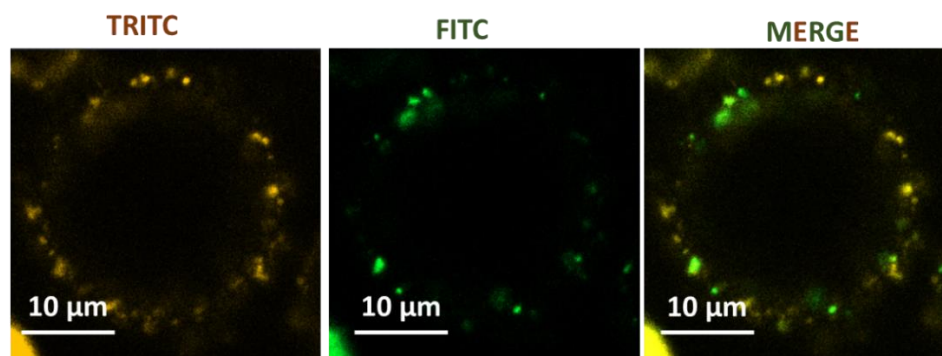


Fig. S24: Representative Two color images of surfactant stabilized o/w interface with GOx-TRITC, ALP-FITC, and untagged HRP in the presence of G-6-P. Experimental condition: [ALP-FITC] = 1 μM , [GOx-TRITC] = 0.5 μM , [HRP] = 0.5 μM , [$\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$] = 250 μM , [G-6-P] = 1 mM, [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

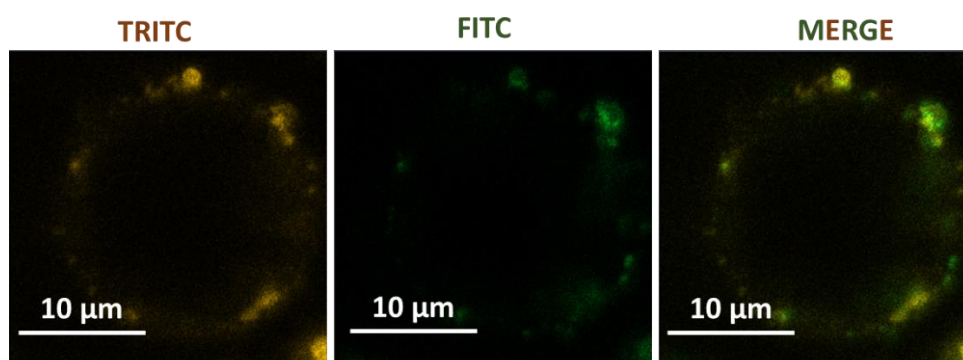


Fig. S25: Representative Two color images of surfactant stabilized o/w interface with GOx-TRITC, ALP-FITC, and untagged HRP in the presence of Glucose and Phosphate. Experimental condition: [ALP-FITC] = 1 μM , [GOx-TRITC] = 0.5 μM , [HRP] = 0.5 μM , [$\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$] = 250 μM , [Glucose] = 1 mM, [Na_2HPO_4] = 1 mM, [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

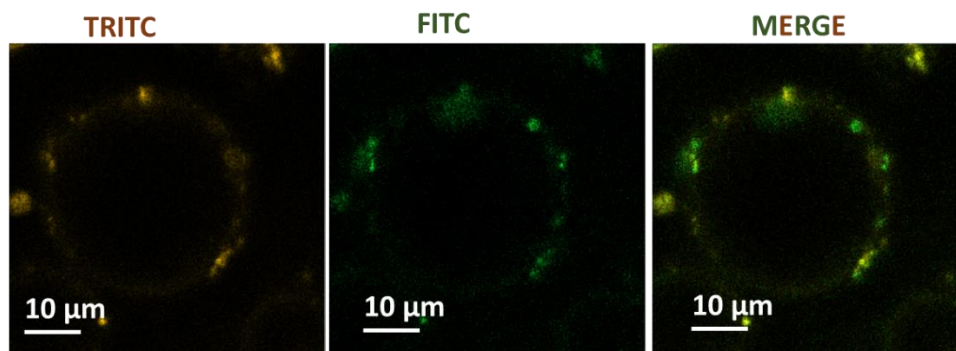


Fig. S26: Representative Two color images of surfactant stabilized o/w interface with GOx-TRITC, ALP-FITC, and untagged HRP in the presence of Phenylphosphate. Experimental condition: [ALP-FITC] = 1 μM , [GOx-TRITC] = 0.5 μM , [HRP] = 0.5 μM , [$\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$] = 250 μM , [Phenylphosphate] = 1 mM, [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

7. HPNPP catalysis in surfactant stabilized o/w interface:

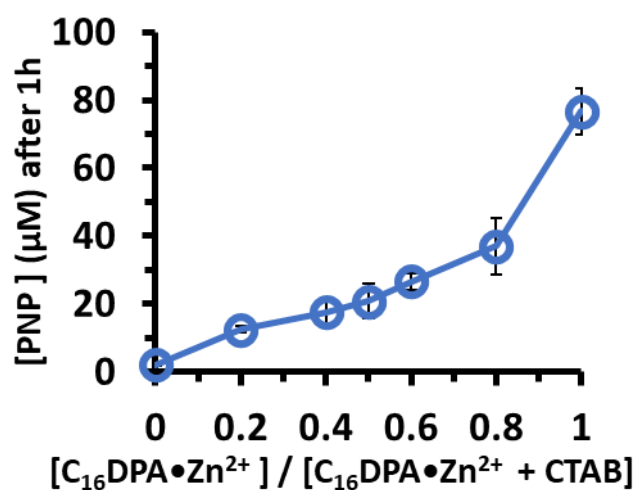


Fig. S27: Representative image of PNP formation as a function of surfactant [$\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$]/[total surfactant conc.] Concentration ratio from 0-1 at fixed HPNPP concentration of 1 mM, and fixed total concentration of surfactant 250 μM . Here we have used a mixture of surfactant $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ and CTAB in the o/w interface to check the catalytic activity of HPNPP. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

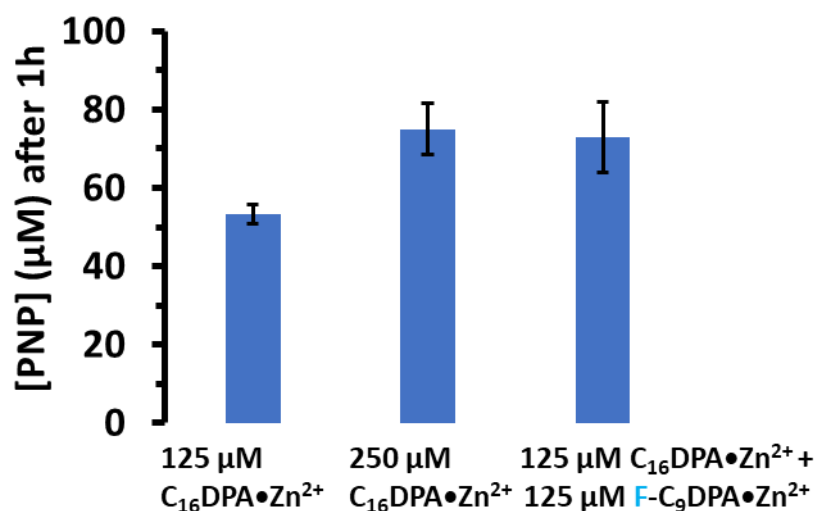


Fig. S28: Representative bar image of PNP formation after 1h in the presence of only 125 μM, 250 μM of C₁₆DPAZn²⁺, and the mixture of 125 μM C₁₆DPA•Zn²⁺ + 125 μM F-C₉DPA•Zn²⁺ in the o/w interface. Here we found that the catalytic ability of this fluorophore-containing surfactant was comparable to C₁₆DPA•Zn²⁺ in the o/w droplet. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

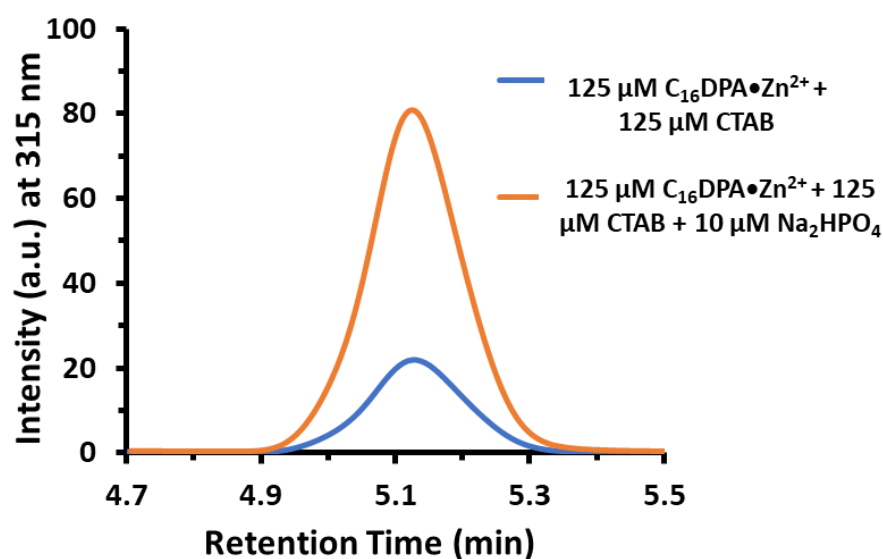


Fig. S29: Representative HPLC Plot of PNP formation after 1h in the presence of 125 μM C₁₆DPA•Zn²⁺ + 125 μM CTAB in the o/w interface. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

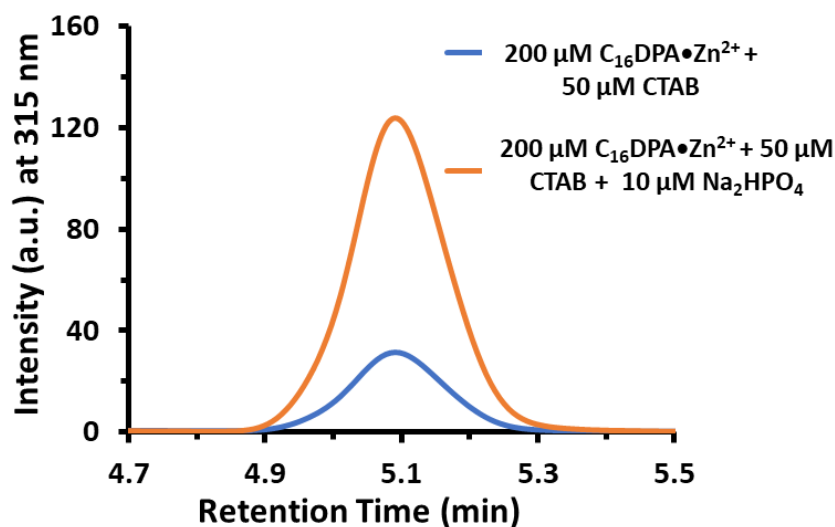


Fig. S30: Representative HPLC Plot of PNP formation after 1h in the presence of 200 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 50 μM CTAB in the o/w interface. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

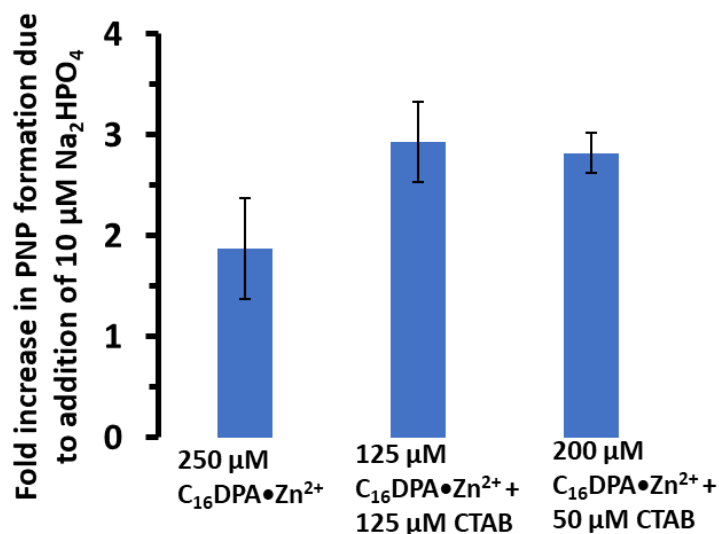


Fig. S31: Representative image of fold increase in PNP formation due to the addition of 10 μM Na_2HPO_4 after 1h in the presence of only 250 μM of $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$, and the mixture of 125 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 125 μM CTAB, and 200 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 50 μM CTAB in the o/w interface. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

Table S1 : Table representing the amount of hydrolyzed p-nitrophenol (PNP) formation after 1 hr in the presence of 250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ and different conc. of Na_2HPO_4 in o/w interface.

System	[PNP] (μM) after 1h
250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 1 mM HPNPP	76 \pm 6.7
250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 1 mM HPNPP + 0.01 mM Na_2HPO_4	167 \pm 25
250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 1 mM HPNPP + 0.1 mM Na_2HPO_4	100 \pm 8
250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 1 mM HPNPP + 01 mM Na_2HPO_4	6 \pm 0.8

Table S2 : Table representing the amount of hydrolyzed p-nitrophenol (PNP) formation after 1 hr in the presence of 250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ and different conc. of NaCl in o/w interface.

System	[PNP] (μM) after 1h
250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 1 mM HPNPP	76 \pm 6.7
250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 1 mM HPNPP + 0.01 mM NaCl	83 \pm 10
250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 1 mM HPNPP + 0.1 mM NaCl	104 \pm 12.5
250 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 1 mM HPNPP + 1 mM NaCl	78 \pm 8.3

8. Fluorescent Microscopic Images of o/w droplet:

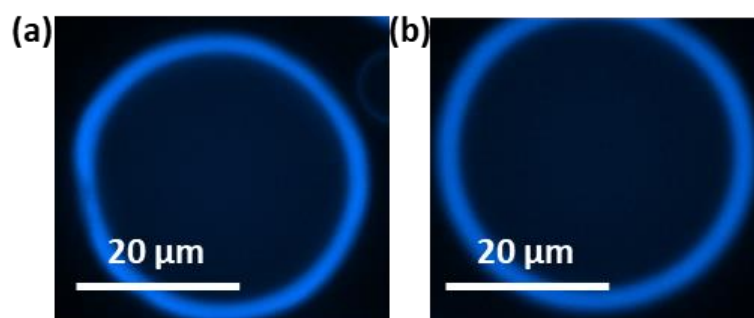


Fig. S32: Fluorescent microscopic images o/w microemulsion where fluorescent but catalytic Zn(II)-metallo-surfactant ($F-C_9DPA \cdot Zn^{2+}$) has been used to probe the surfactant aggregation pattern at the o/w interface in presence of $245 \mu M C_{16}DPA \cdot Zn^{2+} + 5 \mu M F-C_9DPA \cdot Zn^{2+}$. Experimental conditions: [1,2-dichlorobenzene] = $20 \mu l$ in $980 \mu l$ 5mM HEPES buffer pH7 at $25^\circ C$.

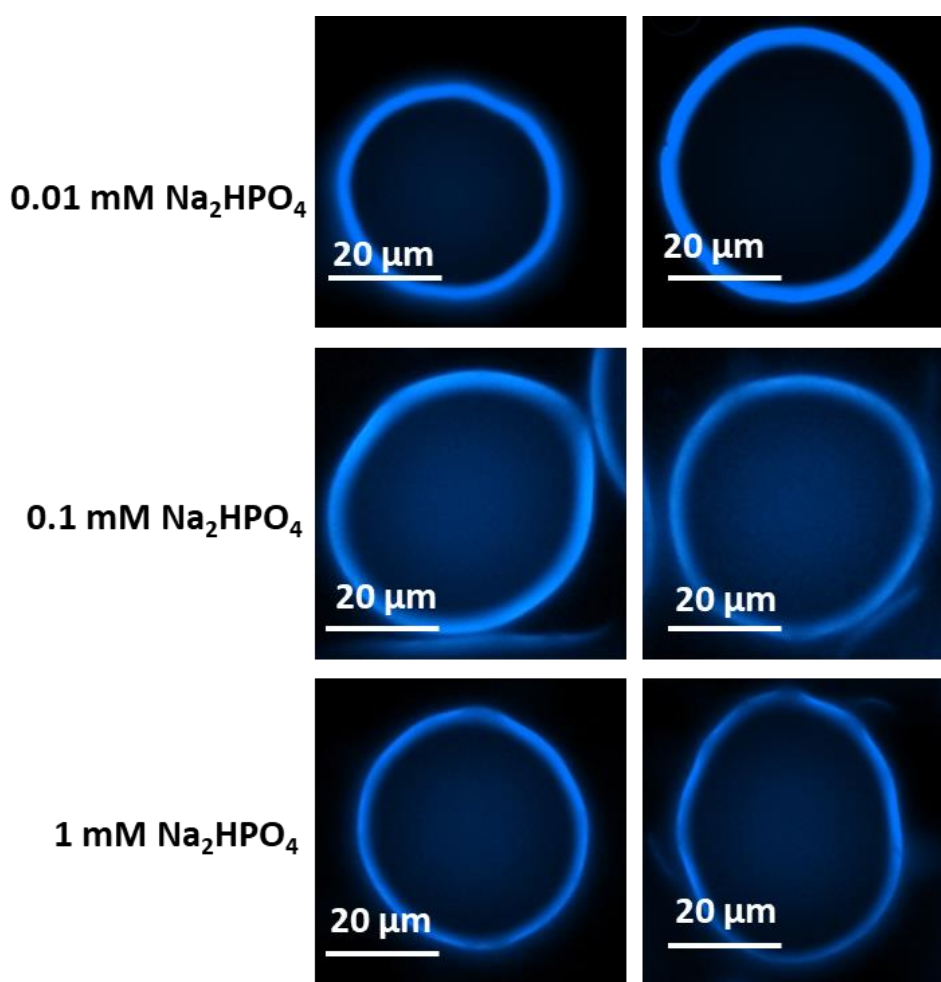


Fig. S33: Fluorescent microscopic images o/w microemulsion where fluorescent but catalytic Zn(II)-metallo-surfactant ($F-C_9DPA \cdot Zn^{2+}$) has been used to probe the surfactant aggregation pattern at the o/w interface in presence of $245 \mu M C_{16}DPA \cdot Zn^{2+} + 5 \mu M F-C_9DPA \cdot Zn^{2+} + 1mM$ HPNPP and different conc. of (0.01 - 1mM) Na_2HPO_4 . Experimental conditions: [1,2-dichlorobenzene] = $20 \mu l$ in $980 \mu l$ 5mM HEPES buffer pH7 at $25^\circ C$.

125 μM C16DPAZn + 125 μM CTAB

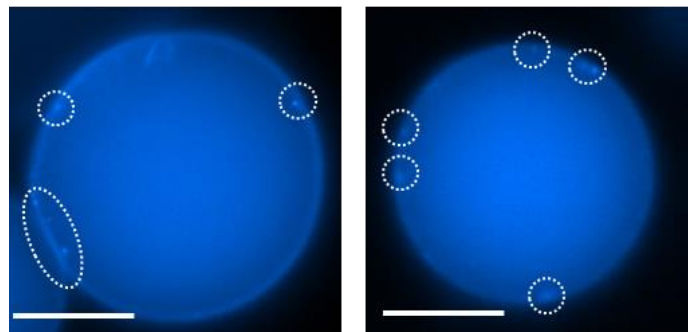


Fig. S34: Fluorescent microscopic images o/w microemulsion where fluorescent but catalytic Zn(II)-metallosurfactant ($\text{F-C}_9\text{DPA}\cdot\text{Zn}^{2+}$) has been used to probe the surfactant aggregation pattern at the o/w interface in presence of 120 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 5 μM $\text{F-C}_9\text{DPA}\cdot\text{Zn}^{2+}$ + 125 μM CTAB. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C. Scale bar here is 20 μm .

**125 μM C16DPAZn + 125 μM CTAB
+ 1 mM HPNPP**

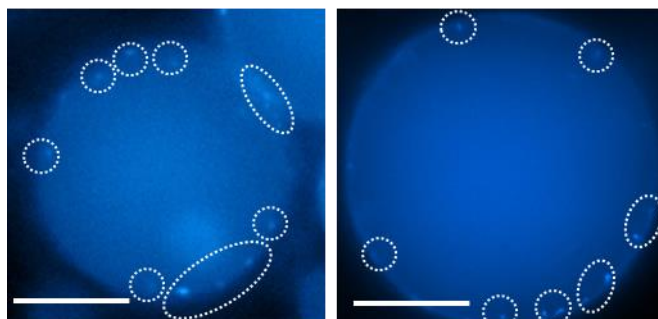


Fig. S35: Fluorescent microscopic images o/w microemulsion where fluorescent but catalytic Zn(II)-metallosurfactant ($\text{F-C}_9\text{DPA}\cdot\text{Zn}^{2+}$) has been used to probe the surfactant aggregation pattern at the o/w interface in presence of 120 μM $\text{C}_{16}\text{DPA}\cdot\text{Zn}^{2+}$ + 5 μM $\text{F-C}_9\text{DPA}\cdot\text{Zn}^{2+}$ + 125 μM CTAB + 1 mM HPNPP. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C. Scale bar here is 20 μm .

**125 μM C16DPAZn + 125 μM CTAB +
1 mM HPNPP + 0.01 mM Na₂HPO₄**

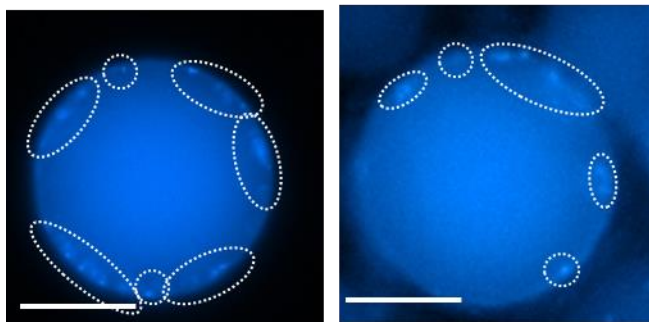


Fig. S36: Fluorescent microscopic images o/w microemulsion where fluorescent but catalytic Zn(II)-metallo-surfactant (F-C₉DPA•Zn²⁺) has been used to probe the surfactant aggregation pattern at the o/w interface in presence of 120 μM C₁₆DPA•Zn²⁺ + 5 μM F-C₉DPA•Zn²⁺ + 125 μM CTAB + 1 mM HPNPP + 0.01 mM Na₂HPO₄. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C. The scale bar here is 20 μm .

**125 μM C16DPAZn + 125 μM CTAB
+ 1 mM HPNPP + 0.1 mM Na₂HPO₄**

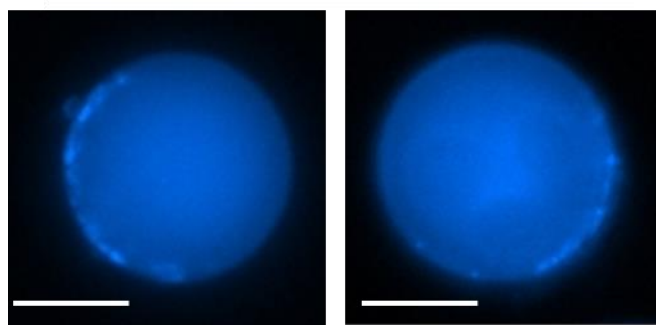


Fig. S37: Fluorescent microscopic images o/w microemulsion where fluorescent but catalytic Zn(II)-metallo-surfactant (F-C₉DPA•Zn²⁺) has been used to probe the surfactant aggregation pattern at the o/w interface in presence of 120 μM C₁₆DPA•Zn²⁺ + 5 μM F-C₉DPA•Zn²⁺ + 125 μM CTAB + 1 mM HPNPP + 0.1 mM Na₂HPO₄. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C. The scale bar here is 20 μm .

**125 μM C₁₆DPAZn + 125 μM CTAB
+ 1 mM HPNPP + 1 mM Na₂HPO₄**

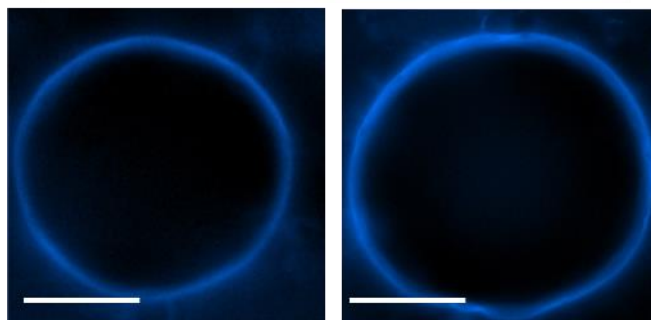


Fig. S38: Fluorescent microscopic images o/w microemulsion where fluorescent but catalytic Zn(II)-metallo-surfactant (F-C₉DPA•Zn²⁺) has been used to probe the surfactant aggregation pattern at the o/w interface in presence of 120 μM C₁₆DPA•Zn²⁺ + 5 μM F-C₉DPA•Zn²⁺ + 125 μM CTAB + 1 mM HPNPP + 1 mM Na₂HPO₄. Experimental conditions: [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C. The scale bar here is 20 μm .

In this case, the band is like only metallosurfactant-doped o/w droplet as shown in Fig. S33, ESI. It is due to present of more phosphate which binds strongly with the metallosurfactant resulting an almost homogeneous interface. However, the band width or intensity is lower in this case as the amount of metallosurfactant is two-times lower than the previous one (Fig. S33).

9. Supporting Videos:

Videos showing clustering of metallosurfactant in the presence of substrate HPNPP or HPNPP + P_i recorded with 100x objective after 2 minutes of drop-casting the solution on the glass slide (played at 2x speed of the original recorded video).

Experimental condition: **For Supporting Video S1**, this video shows control experiment of clustering of metallosurfactant in absence of substrate HPNPP or HPNPP + P_i. [C₁₆DPA•Zn²⁺] = 120 μM , [F-C₉DPA•Zn²⁺] = 5 μM , [CTAB] = 125 μM , [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

For Supporting Video S2, this video shows clusters of metallosurfactant in presence of substrate HPNPP. [C₁₆DPA•Zn²⁺] = 120 μM , [F-C₉DPA•Zn²⁺] = 5 μM , [CTAB] = 125 μM , [HPNPP] = 1 mM, [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

For Supporting Video S3, this video shows clusters of metallosurfactant in presence of substrate HPNPP + P_i. [C₁₆DPA•Zn²⁺] = 120 μM , [F-C₉DPA•Zn²⁺] = 5 μM , [CTAB] = 125 μM , [HPNPP] = 1 mM, [Na₂HPO₄] = 0.01 mM, [1,2-dichlorobenzene] = 20 μl in 980 μl 5mM HEPES buffer pH7 at 25°C.

10. References:

[S1] Priyanka, S. Kaur Brar and S. Maiti, *ChemNanoMat*, 2022, 8, e202100498

[S2] Aastha, Priyanka and S. Maiti, *Chem. Commun.*, 2023, 59, 6536–6539.

[S3] D. V. Kachkin, V. V. Lashkul, N. A. Gorsheneva, S. A. Fedotov, M. S. Rubel, Y. O. Chernoff and A. A. Rubel, *Int. J. Mol. Sci.*, 2023, 24, 14122.