Supporting information

for

Encapsulation of Multiple Enzymes within a Microgel via Water-in-Water Emulsion for Enzymatic Cascade Reaction

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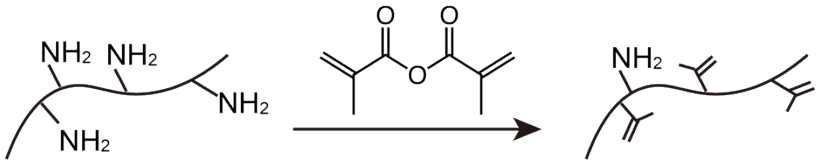
Materials

Gelatin from cold water fish skin, FITC-isothiocyanate (purity=95%), Rhodamine B isothiocyanate (purity~100%), Cy5-NHS (purity=95%), FITC-BSA (quality: 200) and BSA (purity=95%) were purchased from Sigma–Aldrich Co. LLC, USA. Methacrylic anhydride (purity>97.0%) and deuterium oxide (purity=99.8%) were purchased from Tokyo Chemical Industry Co., Ltd, Japan. Horseradish peroxidase (>100 units/mg), D-glucose oxidase from Aspergillus niger (150–250units/mg), β-D-galactosidase from Escherichia coli (>500units/mg) Boronic acid (purity=99.5%), potassium chloride (purity=99.5%), hydrogen chloride, lithium acetate (purity=98.0%), ninhydrin (purity=98.0%), hydrindantin (purity~100%) and sodium hydroxide (purity=97.0%) were purchased from Wako Pure Chemical Industries, Ltd, Japan. PD-10 column was purchased from Global Life Sciences Solutions USA LLC (Cytiva), USA.

Synthesis

All starting materials and solvents were used without further purification (Scheme 1). Nuclear magnetic resonance spectra were run in D_2O with a JEOL ECZ400S 400MHz spectrometer to acquire ^1H NMR spectra. Chemical shifts (δ) are expressed in parts per million and are reported relative to solvent peaks as an internal standard in ^1H NMR spectra. The molecular weight distribution (MWD) curves, number-average molecular weights (M_n), and polydispersity indices ($Đ_M$) were measured by SEC in acetic buffered saline containing 100 mM NaOAc, 300 mM NaCl and 1 mM EDTA・2Na at 25 °C (flow rate = 0.65 mL min^{-1}) with two linear-type PVA gel columns (Shodex SB-803HQ exclusion limit: 1×10^5 g mol^{-1}; particle size: 6 μm; pore size: N/A; 8.0 mm i.d. × 30 cm and Shodex SB-806M HQ; exclusion limit: 2×10^7 g mol^{-1}; particle size: 13 μm; pore size: N/A; 8.0 mm i.d. × 30 cm) and an HPLC (JASCO) instrument equipped with pump (PU-2080Plus), column oven (CO-2060Plus) and refractive index detector. The columns were calibrated against 6 poly(ethylene glycol) standards [PEG: M_p=7.50×10^2 to 5.80×10^6 g mol^{-1}].

Scheme S1



Methacryloyl gelatin (GelMA)

Cold water gelatin (6.00 g) was dissolved in 20 mL of pure water. Methacrylic anhydride (5.16 g, 33.4 mmol) was added dropwise under cooling with a 0 °C ice bath and the resulting

solution was then stirred for 1 h at room temperature. The solution was dialyzed against pure water to remove surplus methacrylic anhydride and by-produced methacrylic acid. The solid GelMA was obtained after freeze drying. GelMA-2 and GelMA-3 were synthesized via same way except the amount of methacrylic anhydride; 2.52 g (16.3 mmol) for GelMA-2 and 1.26 g (8.16 mmol) for GelMA-3.

FITC-HRP

Horse radish peroxidase (10.0 mg) and fluorescein isothiocyanate (1.2 mg, 3.1 μmol) was dissolved in the solution (50 mM, 1.5 mL, pH=8.2).[1] The mixture was stirred for 1 h at 25 °C. Fluorescein labelled horse radish peroxidase was purified by PD-10 column and stocked at 4 °C.

Rhodamine B-GOD

Glucose oxidase (10.0 mg) and rhodamine B isothiocyanate (1.4 mg, 2.9 μmol) was dissolved in the solution (50 mM, 1.5 mL, pH=8.2). The mixture was stirred for 1 h at 25 °C. Rhodamine B labelled glucose oxidase was purified by PD-10 column and stocked at 4 °C.

Dansyl-β-Gal

β-D-galactosidase (10.0 mg) and dansyl chloride ester (1.0 mg, 1.3 μmol) was dissolved in the solution (50 mM, 1.5 mL, pH=8.2). The mixture was stirred for 1 h at 25 °C. Cy 5 labelled β-D-galactosidase was purified by PD-10 column and stocked at 4 °C.

Methods

The determination of residual amino groups in GelMA

Ninhydrin (334 mg, 2.24 mmol) and hidrindantin (60.6 mg, 0.188 mmol) were dissolved in 15 mL of deoxygenated dimethyl sulfoxide. Lithium acetate aqueous solution (5mL, 4M) was added to the ninhydrin solution. The fish derived gelatin was dissolved in ultra-pure water with the concentration of 1.4, 1.2, 1.0, 0.8, 0.6, 0.4 g/L for collecting calibration curve. The 200 μL of gelatin solution and 200 μL of ninhydrin solution were mixed and kept 95 °C for 10 min. Ethanol/water mixed solvent (1/1, v/v) was immediately poured to the solution and the mixture was cooled with iced bath. The absorbance at 570 nm was evaluated by UV/Vis spectrometer (V-730, JASCO, Japan), providing calibration curve between gelatin concentrations and absorbance. The aqueous solution of GelMAs were evaluated in the same way and residual amino groups were calculated from the calibration curve.

Fabricating enzyme(s) loaded microgels

The PBS solution of GelMA (225 μL, 20 wt%), PVP (900 μL,10 wt%) and photo initiators 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (9 μL, 16 mM) and tri-ethanolamine (13.5 μL, 4M) were added glass tube and vigorously mixed for 30 seconds at 25 °C, giving W_{GelMA}/W_{PVP} emulsion. The emulsion was passed through glass filter with the pore size of 5 or 10 μm. In the case of single or double enzymes loading, each enzyme aqueous solution (13.5 μL, 5 mg/mL) was added to the emulsion and the emulsion was mixed by inverting the glass tube for 10 min. In the case of three enzymes loading, the amount of each enzyme solution was changed to 9.0 μL (5 mg/mL). The emulsion including enzymes were exposed to UV irradiation; the Xe lamp (UI-502Q, Ushio Inc. Japan) with 360 nm (±40 nm) sharp-cut filter for 20 seconds. The PVP solution consisting of enzymes loaded microgels were diluted by 1 mL of pure water and the microgels were collected by using centrifuge with 30,000 G at 10 °C (Allegra X-30, Beckman Coulter, Germany). The collected microgels were redispersed in pure water to wash out PVP and recentrifuged. This process was repeated for three times.

Fluorescence microscope

Fluorescence images of W_{GelMA}/W_{PVP} including fluorescent labelled gelatin and enzymes loaded microgels were confirmed by fluorescence microscope (BZ-X810, Keyence, Japan) at a magnification of 40x or 100x objective lenses with excitation wavelength was selected for FITC. The samples were applied on the glass-base dish (Iwaki, Japan).

Microgel size measurement

The particle size of microgels were determined from fluorescence microscopic images.

ImageJ software converted the original images to 8-bit black and white ones, adjusted auto threshold and ran particle analysis program with sphericity > 0.90.[1] The obtained spherical areas were converted to diameters by following equation;

$$D = \left(\frac{A}{\pi}\right)^{0.5} \times 2$$

where, D and A are calculated diameter of microgel, and spherical area determined by ImageJ. Additionally, obtained diameter histograms of the microgels were fitted as lognormal distribution by *MATLAB* (version: R2022a, The Math Works, Inc., USA, www.mathworks.com/).

CLSM observations of microgels

The obtained microgels loading fluorescent labelled enzymes i.e., FITC-HRP, Rhodamine B-GOD and/or Cy5-β-Gal, put on the glass-based dish (Iwaki, Japan). The images of microgels were acquired by CLSM (Eclipse Ti; Nikon, Japan) at a magnification of 60× with an excitation wavelength of 488, 561 and 640 nm.

Determination of encapsulation efficiency of enzymes to the microgels

The PBS solution of GelMA (225 μL, 20 wt%), PVP (900 μL, 10 wt%) and photo initiators 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (9 μL, 32 mM) and tri-ethanolamine (13.5 μL, 4M) were added glass tube and vigorously mixed for 30 seconds at 25 °C, giving W_{GelMA}/W_{PVP} emulsion. The emulsion was passed through glass filter with the pore size of 5 or 10 μm. FITC-BSA, FITC-HRP, RhodamineB-GOD and/or Cy5-β-Gal solutions (13.5 μL, 5 mg/mL) were added to the emulsion and the emulsion was mixed by inverting the glass tube for 10 min. In the case of three enzymes (FITC-HRP, RhodamineB-GOD and/or Cy5-β-Gal) simultaneously added, the amount of each solution was changed to 9 μL (5 mg/mL). The given emulsions were centrifuged at 10 °C to separate GelMA and PVP phase. The amounts of enzymes existing in each phase were determined by fluorescence spectrometer (FP-8350, JASCO, Japan). FITC-BSA and FITC-HRP were detected at 420 nm with the excitation wavelength 494 nm. Rhodamine B-GOD was detected at 580 nm with the excitation wavelength 550 nm. Dansyl-β-Gal was detected at 340 nm with the excitation wavelength 485 nm. The slit width was set to 5 nm for the excitation and 5 nm for the emission.

The HRP and GOD cascade reaction inside the microgel

The HRP and GOD loaded microgel was suspended in 1315 μL of citrate buffered saline at pH = 5.35 with the concentrations: [HRP]=1.17 μg/mL,[GOD]=1.24 μg/mL. The citrate buffered saline of OPD (75 μL, 2.78 mM) was added to the suspension. The cuvette was stabilized at 30 °C for 15 min. After that, the solution of D-glucose (100 μL, 1.00 M) was added to the cuvette.

The reaction was pursuit by measuring absorbance at 440 nm by UV/Vis spectrometer (V-730, JASCO, Japan).

The HRP, GOD and β-Gal cascade reaction inside the microgel

The HRP, GOD and β-Gal loaded microgel was suspended in 1115 μL of phosphate buffered saline at pH = 6.60 with the concentrations: [HRP]=0.51 μg/mL, [GOD] =0.65 μg/mL, [β-Gal]= 1.08 μg/mL. The phosphate buffered solution of OPD (75 μL, 2.78 mM) was added to the suspension. The cuvette was stabilized at 30 °C for 15 min. After that, the solution of lactose (300 μL, 0.33 M) was added to the cuvette. The reaction was pursuit by measuring absorbance at 440 nm by UV/Vis spectrometer (V-730, JASCO, Japan).

Supplementary Figures

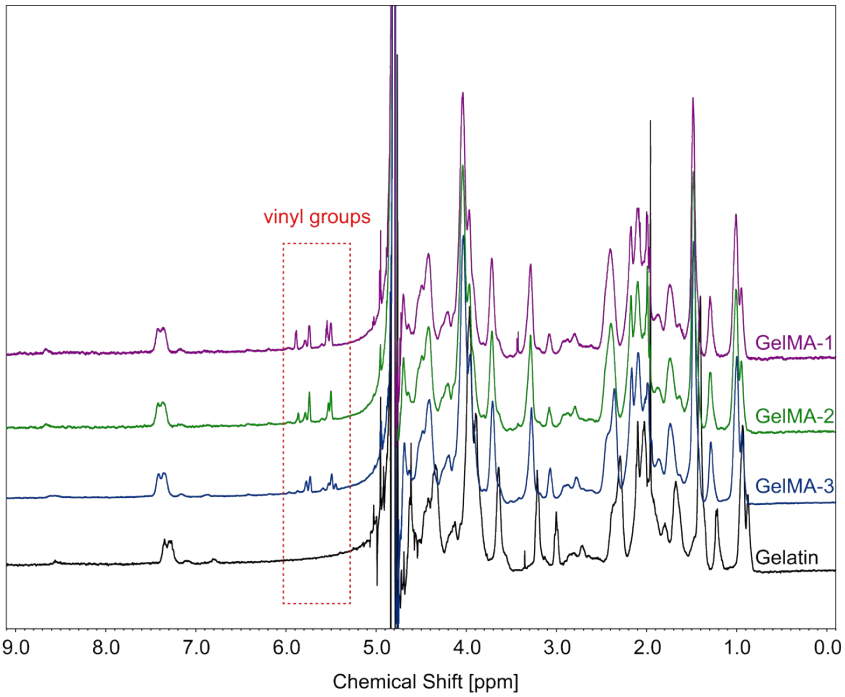


Figure S1. ^{1}H NMR spectrum of GelMA-1, GelMA-2, GelMA-3 and naked cold water fish gelatin in D_2O (400 MHz, 30 °C).

Figure S2. Size-exclusion chromatograph of GelMA-1 and naked cold water fish gelatin.

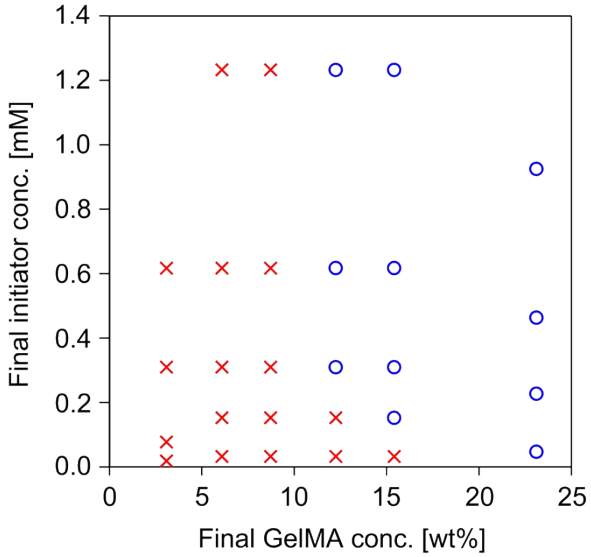


Figure S3. Gelation behavior of GelMA-1 depending on the final concentrations of GelMA-1 and photo initiator; blue circle: gelated and red cross: not gelated.

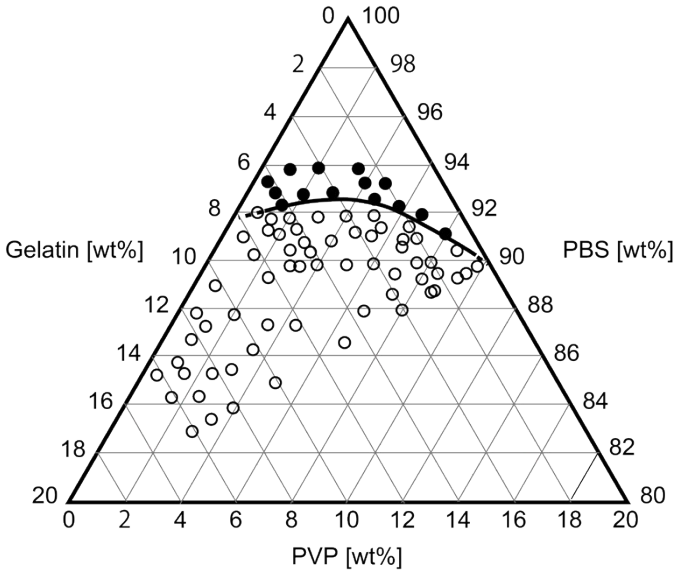


Figure S4. The ternary phase diagram of GelMA-1/PVP/PBS at 25 °C; white circle is phase separated composition and black circle is homogeneous mixture.

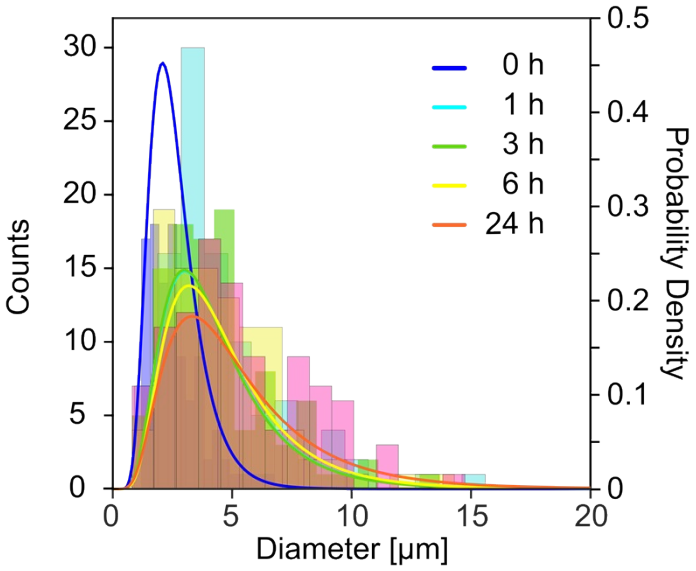


Figure S5. The size distribution of microgels 0, 1, 3, 6 and 24 h after passing through microporous glass filter with a pore size of 5 μm.

Table S1. The effect of the pH of the emulsion on protein partitioning against GelMA phase with GelMA/PVP = 3/7 (v/v).

	IEP	pH 7.4	pH 5.4
BSA	5.0	89%	70%
HRP	7.2	82%	86%
GOD	4.2	82%	71%

Table S2. The effect of mixing ratio of GelMA and PVP on protein partitioning against GelMA phase.

GelMA/PVP	4/6	3/7	2/8
BSA	93%	89%	97%
HRP	69%	82%	97%
GOD	74%	89%	96%

Table S3. The effect of the amount of protains added to the emulsion on protein partitioning against GelMA phase.

	GelMA/PVP = 3/7		
	50 μg/mL	100 μg/mL	150 μg/mL
BSA	86%	89%	94%
HRP	87%	82%	82%
GOD	87%	89%	87%

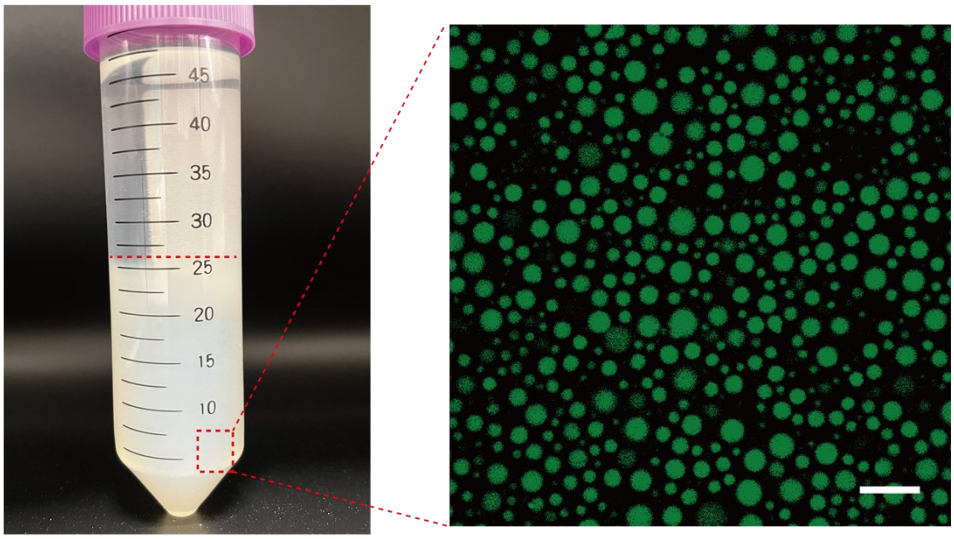


Figure S6. The large scale production of FITC-BSA-loaded microgels. The centrifuged microgels (left) and their image by confocal laser scanning microscope (right).

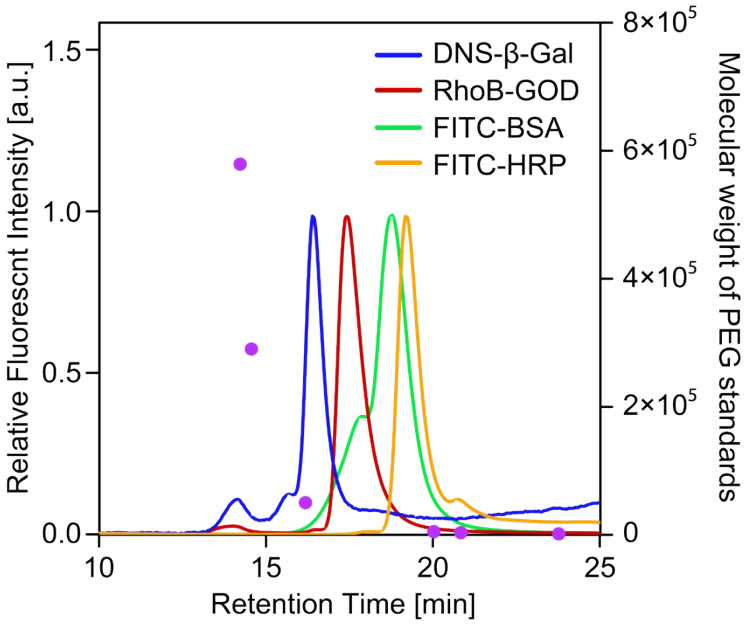


Figure S7. The size exclusion chromatograms of fluorescence labelled proteins (color lines, left vertical axis) and PEG standards (purple dots, right vertical axis).

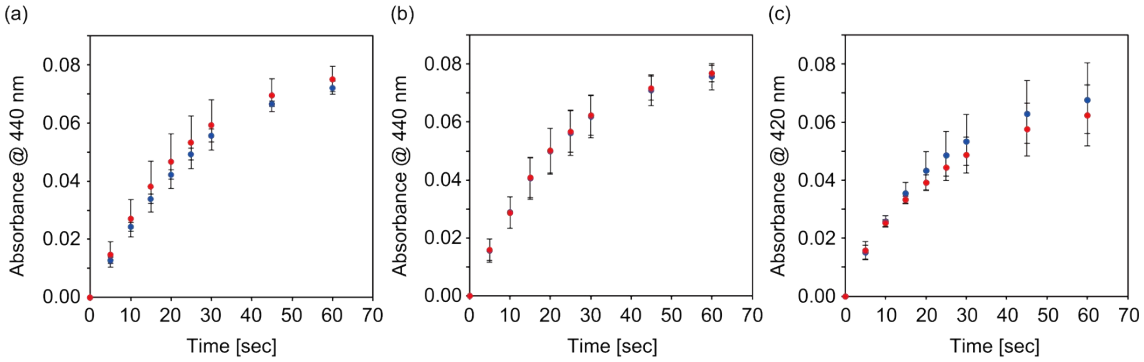


Figure S8. The effect of UV irradiation against enzymes' activities. Time course of products generated by (a) HRP, (b) GOD and (c) β-Gal before (blue) and after (red) UV irradiation at 360 nm for 20 sec.

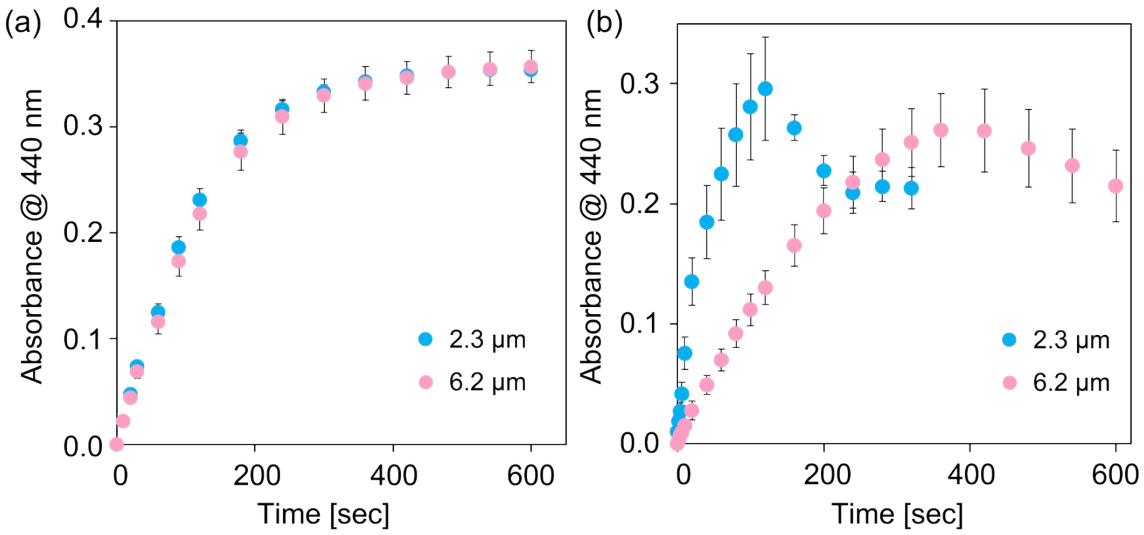


Figure S9. (a) The enzymatic reaction contributed by HRP inside the microgel particles with different particle size. Time course of the absorbance at 440 nm, which corresponds to oxidized *o*-phenylenediamine (OPD) in citrate buffer at 30 °C and pH= 5.35. Concentrations: [HRP]=0.54 μg/mL, [OPD]=0.12 mM. (b) The enzymatic reaction contributed by GOD inside the microgel particles with different particle size and HRP outside of the microgel. Time course of the absorbance at 440 nm, which corresponds to oxidized OPD in citrate buffer at 30 °C and pH= 5.35. Concentrations: [GOD]=0.81 μg/mL [HRP]=6.67 μg/mL, [glucose]=6.67 mM, [OPD]=0.16 mM. All error Bars; SD (n=3).

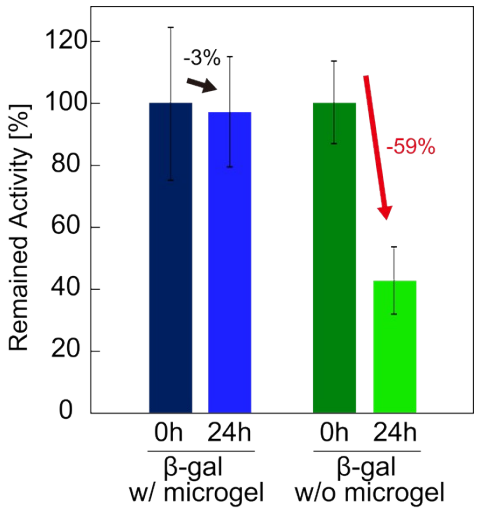


Figure S10. The activity of β-Gal with or without microgels in the citrate buffer with pH = 6.0 at 37 °C after 24 hours. The β-Gal inside the microgels was protected from surrounding environment.

References

(1) Schneider, C.A., Rasband, W.S., Eliceiri, K.W., NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **2012**, *9*, 671-675.

(2) Zhao, W.; Hu, J.; Gao, W. Glucose Oxidase–Polymer Nanogels for Synergistic Cancer-Starving and Oxidation Therapy. *ACS Appl. Mater. Interfaces* **2017**, *9* (28), 23528–23535. https://doi.org/10.1021/acsami.7b06814.