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Engineering Metabolic cycle-inspired Hydrogels with Enzyme-Fueled Programmable Transient Volume Changes

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Materials and instrumentations

N-(2-Hydroxypropyl)methacrylamide (HPMA), *N,N'*-methylenebismethacrylamide (mBIS), methacrylic acid stabilized with MEHQ (MA), and ammonium persulfate (APS) were obtained from Tokyo Chemical Industry Co., Ltd. Tokyo Japan. *N, N, N', N'*-Tetramethylethylenediamine (TEMED) and trypsin from porcine pancreas were acquired from Sigma Aldrich, St. Louis, Mo. Acrylamide fluorescein (AF) from the previous research was used.[54] Lysozyme hydrochloride from Egg White (MW: 14 kDa), activated aluminium oxide, dimethyl sulfoxide, hydrochloric acid and calcium chloride were obtained from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. Dialysis tube (MWCO 3,500) was picked up from Fisher bland, Inc. Fluorescence spectra was measured using FP-8500 (Jasco Ltd., Tokyo, Japan). MALDI-TOF-MS analysis was done through Autoflex III (Bruker Daltonics, Bremen, Germany). Compression test was done using EZ Test (Shimadzu Corp., Kyoto, Japan) and the results were analyzed through TRAPEZIUM2 software.

1. Metabolic-cycle inspired hydrogel synthesis

Synthesis of AcTryp

Acrylamide trypsin (AcTryp) used for polymerization was synthesized through a protein conjugation reaction with slight changes from the methodology reported earlier[50]. Amine units of lysine were modified with an *N*-hydroxysuccinimide ester-activated compound with lysine residues. In 20 mL of 100 mM phosphate buffer (pH 7.5), trypsin (200 mg, 0.009 mmol) and benzamidine dihydrochloride (13 mg, 0.08 mmol) were dissolved. *N*-hydroxysuccinimide ester (37 mg, 0.22 mmol) was dissolved in 1 mL of DMSO and added dropwise in the solution at 25 ℃ for 90 min. The solution was dialyzed (MWCO: 3,500 Da) against aqueous 1 mM HCl with 10 mM $CaCl_2$ for a day, then in MilliQ for a day at 4 ℃. Subsequently, the obtained solution was filtered (pore size: 0.4 μm) and lyophilized to produce a white powder with 89.9% yield.

Characterization of AcTryp by MALDI-TOF-MS

MALDI-TOF-MS was used to examine the number of conjugated acrylate groups of the synthesized trypsin[61]. The aqueous solution of the conjugate (1.0 mg mL^{-1}) was mixed proportionally with the matrix (8 mg sinapic acid in 1 mL water/MeCN (50:50 in volume)). 2 μL of the mixture was then spotted on the plate and dried. The number of conjugated acrylate groups was determined through the molecular weight comparison between conjugate trypsin to native trypsin. The synthesized acrylamide trypsin showed higher molecular weight when compared with native trypsin (Figure S1A). Mass increase of 391 (*m/z*), 505 (*m/z*), and 559 (*m/z*) were determined, accordingly, in the detectable peaks. These correspond to the conjugation of 7, 9, and 10 acrylates on single trypsin when assuming that 1 acrylate is 54.01 (*m/z*).

Characterization of AcTryp by fluorospectrometer

The stained trypsin by fluorescamine was used to quantify the number of conjugated acrylate groups. 0.1 mg mL^{-1} of native trypsin and 0.1 mg mL^{-1} of AcTryp in 10 mM $NaHCO_3$ (pH 8.5) were each mixed with 10 vol% of fluorescamine solution (0.5 mg mL^{-1} in DMSO). The samples were incubated for 15 min at room temperature. The fluorescence intensity of the mixture was measured by the fluorescent spectrometer while setting the excitation wavelength at 395 nm and the emission wavelength at 495 nm. The intensity of AcTryp was 40%, indicating the conjugation of 7 acrylates on single trypsin on average (Figure S1B).

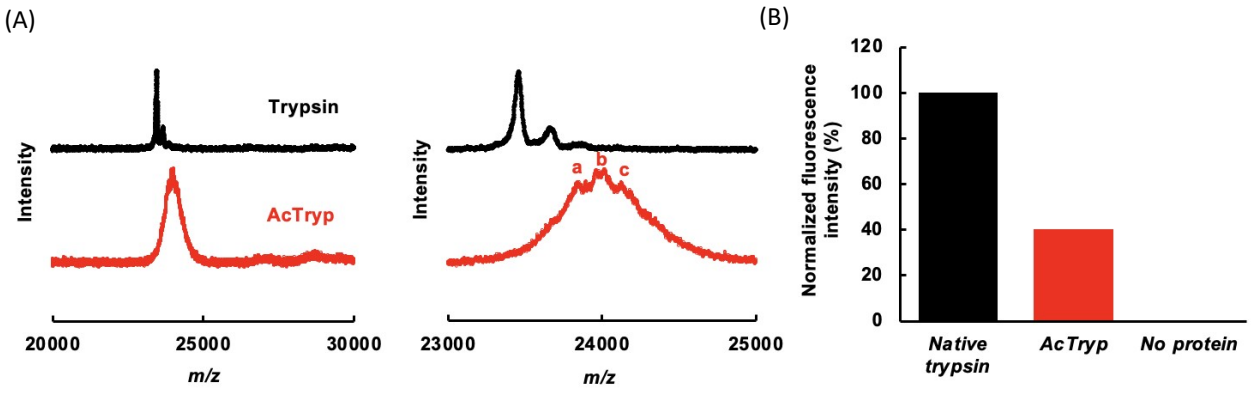

Figure S1. (A) MALDI-TOF-MS analysis of trypsin before (black) and after (red). (B) Fluorescence intensity of the mixture with fluorescamine and native trypsin, AcTryp.

Synthesis of metabolic cycle-inspired hydrogel

Monomethyl ether of hydroquinone (MEHQ) in the commercially available methacrylic acid was removed using aluminum oxide filtration. The gel was synthesized by the free radical co-polymerization of *N*-(2-hydroxypropyl)methacrylamide (200 g L^{-1}, 200 mg), *N, N'*-methylenebismethacrylamide (10 g L^{-1}, 10 mg), methacrylic acid (400 g L^{-1}, 400 mg), and acrylamide trypsin (4 g L^{-1}, 4 mg). A small amount of acrylamide fluorescence (0.5 g L^{-1}, 0.5 mg) was also used for visualization. The co-monomers were dissolved in water (0.5 mL) and degassed to remove the oxygen through nitrogen bubbling in an ice bath for 10 min. The addition of ammonium persulfate (10 g L^{-1}, 10 mg) and *N, N, N', N'*-tetramethylethylenediamine (10 g L^{-1}, 10 mg) to the co-monomer solution started the polymerization. The solution was then immediately loaded on to the glass plates with a silicon spacer (50 mm width, 0.1 mm thickness, and 50 mm length). The polymerization was kept for 30 min at 25 ℃. The pH of the co-monomer solution was adjusted to 3.0, and 10 mM of $CaCl_2$ was added to prohibit denaturation of trypsin during the radical polymerization. The gels, after gelation, were washed with MilliQ and stored at 4 ℃. The hydrogels functionalized with either MA or AcTryp alone were prepared for comparison (Figure S1C).

	HPMA (mmol)	mBIS (mmol)	MA (mmol)	AcTryp (μmol)
EFM gel (MA and AcTryp)	1.39	0.05	4.65	0.18
MA	1.39	0.05	4.65	-
AcTryp gel	1.39	0.05	-	0.18

Figure S1. (C) Chemical composition of the gel.

The programmability of the TVPT at fixed fuel concentration was validated by varying the chemical composition of the gel through adjustments in MA or AcTryp. The same synthesis steps were taken as before but by adding none, 200, and 400 mg of MA at fixed 4 mg AcTryp to determine the forward pathway. Respectively, the feedback pathway was evaluated by varying 1, 2, and 4 mg of AcTryp at a fixed amount of 200 mg MA.

2. TVPT Evaluation

The gel was initially hydrated in 10 mM $NaHCO_3$ buffer (pH 8.5) for three days at 4 ℃. The hydrogel was cut into a circle (8 mm in diameter, 0.3 mm in thickness) before being immersed in 1 mL of 10 mM $NaHCO_3$ (pH 8.5) containing 4 g L^{-1} (4 mg mL^{-1}) of lysozyme at 25 ℃. The transient volume changes of the gels were evaluated as a function of time. For comparison, hydrogels functionalized with either MA alone (MA gel) or AcTryp alone (AcTryp gel) were investigated, as shown below. By measuring the area ratio using Image J, the transient volume changes were assessed, assuming isotropic shrinkage/swelling. [62]

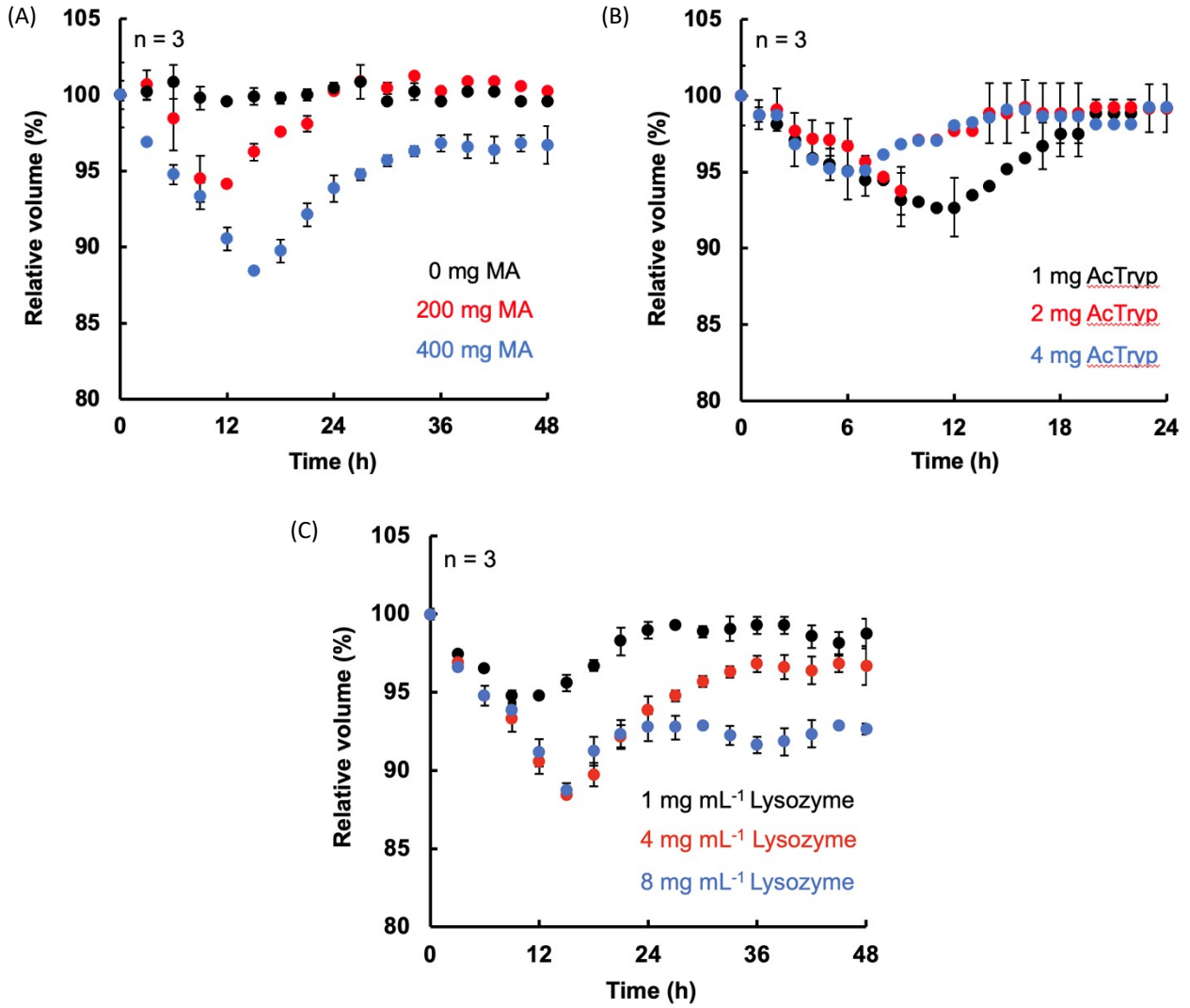

Figure S2. Volume shrinkage of different gels: (A) MA gel with no MA (black), 200 mg MA (red), and 400 mg MA (blue) at a constant 4 mg of AcTryp. (B) AcTryp gel with 1 mg AcTryp (black), 2 mg AcTryp (red), and 4 mg AcTryp (blue) while maintaining a fixed 200 mg of MA. (C) EFM gel in response to 1 mg mL^{-1} (black), 4 mg mL^{-1} (red), and 8 mg mL^{-1} (blue) lysozyme solution.

3. Lysozyme activity assay of the supernatant after TVPT

Freeze-dried *Micrococcus lysodeikticus* cells were suspended at 200 mg mL^{-1} in 10 mM $NaHCO_3$ buffer (pH 8.5). The supernatant solution containing lysozyme (10 µL) was added to *Micrococcus lysodeikticus* cell stock solution to measure the decrease in absorbance at 450 nm with steering at 400 rpm using UV-vis spectra. The decrease in absorbance for the first 40 seconds was used to measure the lysozyme activity. Relative enzymatic activity regarding reaction rate was defined as slope and was used to quantify the active lysozyme present in the supernatant.

The 200 and 400 mg of MA gels showed a 30.8% and 26.1% decline in active lysozyme, respectively (Figure S3A). The increased number of MA showed improved encapsulation of lysozyme for charge stabilization during TVPT. The number of active lysozymes was reduced to 58.4%, 34.7%, and 17.8%, accordingly, with 1, 2, and 4 mg of AcTryp (Figure S3B). The result indicated that the increased amount of AcTryp hydrolyzes lysozyme more effectively during the transition.

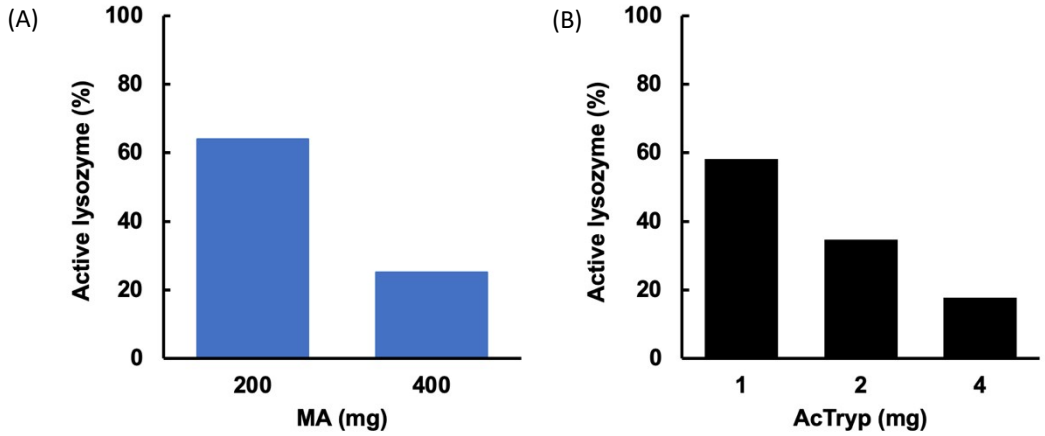

Figure S3. Relative lysozyme activity of supernatants after TVPT of gels with (A) varied amounts of MA at fixed AcTryp (4 mg) and (B) varied amounts of AcTryp at fixed MA (200 mg).

Three different types of gels were subjected to lysozyme assays. The synthesis procedure was consistent across all gels, but there were specific variations: the AcTryp gel was prepared without the addition of MA, and the MA gel was prepared without AcTryp. EFM gel, with both functionalities, contained 400 mg of MA and 4 mg of AcTryp. The results of the assays revealed a clear downward trend in the percentage of active lysozyme concerning different functionalities.

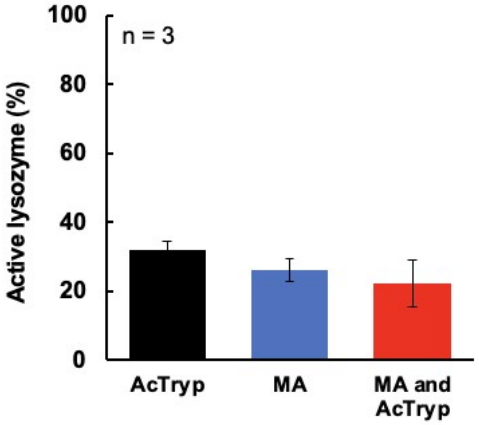

Figure S4. Relative lysozyme activity of supernatants after TVPT of three distinct gel types: gel with AcTryp alone, gel with MA alone, and gel with both MA and AcTryp (EFM gel).

4. SDS-PAGE analysis after TVPT

100 μL of intact lysozyme (4 mg mL^{-1}) and 100 μL of the supernatant after TVPT cycle were lyophilized and dissolved in 50 μL of SDS-PAGE sample buffer, which contains the reducing agent. 500 μL of SDS-PAGE sample buffer was added to the gel disk after TVPT to elute the remaining components inside the gel. All samples were heated for 10 min at 95 ℃ and further incubated for 15 h at r.t. followed by centrifugation at 5 kG for 5 min. After the centrifugation, supernatants were collected. Supernatants were diluted 10 times using SDS-PAGE sample buffer and heated for 10 min at 95 ℃ before the electrophoresis. SDS-PAGE was performed on precast Mini-PROTEIN TGX gel (Tris-glycine-SDS running buffer). 10 μL of the standard and 5 μL of samples were applied on SDS-PAGE gel, and the electrophoresis was performed at constant voltage (200 V) for approximately 70 min. The SDS-PAGE gel was stained with Coomassie Brilliant Blue R-250 and de-stained with the aqueous solution containing 5% methanol and 7.5% acetic acid overnight, respectively.

5. Transient changes in Young's modulus via TVPT

Young's modulus of a hydrogel was measured by finding the relationship between a force applied to the hydrogel and the resultant deformation of the hydrogel. Unlike the previous synthesis methods, the co-monomer solution was loaded onto the glass plates with a silicon spacer (50 mm width, 1 mm thickness, and 50 mm length) for mechanical property detection. The gels were initially hydrated in 10 mM $NaHCO_3$ buffer (pH 8.5) for five days at 4 ℃. The dimension of the EFM gel, with both MA and AcTryp, was 1.9±0.1 mm in thickness and 8.3 ±0.1 mm in diameter after hydration. At the same time, the MA gel showed 3.69±1.2 mm in thickness and 7.4±0.1 mm in diameter. The gels were cut into circles (8 mm in diameter, thickness varied upon chemical composition) and were incubated in 10 mM $NaHCO_3$ buffer (pH 8.5) containing 4 mg mL^{-1} of lysozyme at 25 ℃, accordingly in time. Three gels were tested each day for a week. During the compression test, the speed of the crosshead was 0.5 mm/min, and the load cell was 500 N. The compressive strain reached 40%, and Young's modulus was measured at a strain of 30%. Each compression test was repeated three times, and the error bars indicate the variation of these data. Gels after washing with 10 mM $NaHCO_3$ buffer (pH 8.5) were also tested, followed by the 7 days incubation (Figure S5).

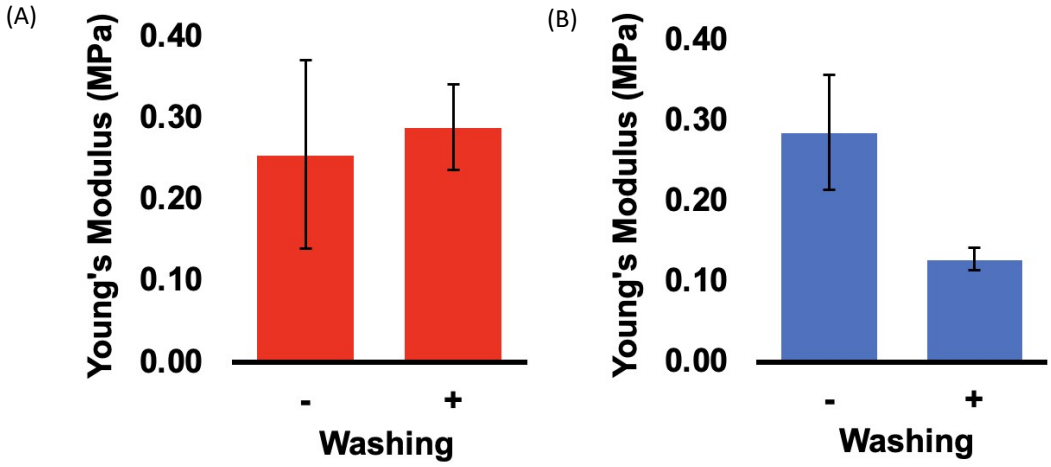

Figure S5. Changes in Young's Modulus after washing (A) EFM gel and (B) MA gel.

References

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