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Enzyme- and Photo-Gated Polymerization Using Readily Available Enzyme-Responsive Fluorescent Dyes

Kanta Ogura, Masahiko Nakamoto*, Michiya Matsusaki*

Table of Contents

1. Materials and methods.

2. General procedure for evaluating the rate of conversion of calcein AM into calcein via esterase-mediated hydrolysis.

3. General procedure for carboxylesterase-responsive PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by calcein AM.

4. General procedure for light-controlled reaction of carboxylesterase-responsive PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by calcein AM.

5. General procedure for light-controlled PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by calcein AM upon in situ addition of carboxylesterase.

6. General procedure for studies on carboxylesterase selectivity in enzyme-responsive PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by calcein AM.

7. General procedure for enzyme-responsive PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by various enzyme-responsive photocatalysts and corresponding enzymes.

8. General procedure for hydrogel synthesis via PET-RAFT co-polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) and *N*,*N*'-methylenebismethacrylamide (mBIS) mediated by various enzyme-responsive photocatalysts and corresponding enzymes.

1. Materials and methods.

Materials

The following chemicals were purchased from commercial sources and used as received unless otherwise indicated: *N*-(2-Hydroxypropyl)methacrylamide, calcein (mixture of isomers), and *N,N*'-methylenebismethacrylamide were purchased from TCI (Tokyo, Japan). 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid, Dulbecco's phosphate-buffered saline, esterase from *Bacillus subtilis*, alkaline phosphatase from bovine intestinal mucosa, and fluorescein di-β-D-galactopyranoside were purchased from Sigma-Aldrich. L (+)-Ascorbic acid and D-PBS (without Ca and Mg, Powder) were purchased from Nacalai Tesque (Kyoto, Japan). Fluorescein di-β-D-glucopyranoside was purchased from Thermo Fisher Scientific. β-Glucosidase was purchased from Toyobo (Osaka, Japan). Calcein-AM was purchased from DOJINDO (Kumamoto, Japan). β-D-Galactosidase, fluorescein diphosphate tetraammonium salt, and dimethyl sulfoxide (DMSO) were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). A light-emitting diode (LED) illuminator (TH2-140X105BL) was purchased from CCS (Kyoto, Osaka, Japan).

Instrumentation

Size-exclusion chromatography (SEC) analysis was performed using an HLC-8320 GPC Eco-SEC instrument equipped with a two-column series configuration (TSKgel SuperMultiporePW-M and TSKgel SuperOligoPW) and TSKgel SuperMP(PW)-M guard columns (TOSOH, Tokyo, Japan). The SEC analyses were performed at a flow rate of 1.0 mL/min by injecting 10 µL of a polymer solution in 200 mM phosphate buffer (pH 7.0). The SEC system was calibrated using a polyethylene glycol/polyethylene oxide standard (Sigma-Aldrich). All SEC samples were previously filtered through a 0.22 µm filter. Proton nuclear magnetic resonance (^{1}H NMR) spectra were acquired using a JNM-ECS400 NMR spectrometer (JEOL, Tokyo, Japan) using DMSO-d_6 or D_2O as the deuterated solvent. Fluorescence spectra were measured using a spectrofluorometer FP-8500 (Jasco, Tokyo, Japan). Photopolymerization was carried out in a reaction vessel in which the reaction mixtures were irradiated with an LED illuminator (TH2-140X105BL). Samples were irradiated with 470 nm blue light at an intensity of 6 mW/cm^2.

2. General procedure for evaluating the rate of conversion of calcein AM into calcein via esterase-mediated hydrolysis.

A solution consisting of PBS (990 µL), DMSO (10 µL), calcein AM (0.25 mg, 2.5×10^{-4} mmol), and carboxylesterase (1 mg) was prepared in a 9 mL glass vial. The vial cap was screwed on lightly, and the vial was left in the incubator at 37 °C for 3 h. Three hours later, the reaction solution was diluted 2500-fold with PBS, and the diluted solution was placed in a cuvette for fluorescence analysis (sensitivity: medium, λ_{ex} = 490 nm, room temperature). A calibration curve (λ = 515 nm) was constructed by measuring the fluorescence (sensitivity: medium, λ_{ex} = 490 nm, room temperature) of 0–100 nM calcein in PBS. Using the calcein calibration curve, the rate of calcein AM conversion into calcein was calculated from fluorescence intensity measurements.

3. General procedure for carboxylesterase-responsive PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by calcein AM.

A solution consisting of PBS (990 μL), DMSO (10 μL), calcein AM (0.125 mg, 12.5×10^{-4} mmol; 0.25 mg, 25×10^{-4} mmol; or 0.5 mg, 50×10^{-4} mmol), and carboxylesterase (1 mg) was prepared in a 9 mL glass vial. The vial cap was screwed on lightly, and the vial was left in the incubator at 37 °C for 3 h. Three hours later, HPMA (71.6 mg, 0.50 mmol) and CPADB (0.35 mg, 1.25×10^{-3} mmol; 0.70 mg, 2.5×10^{-3} mmol; or 1.4 mg, 5.0×10^{-3} mmol) were added to the vial, and the solution was irradiated with blue LED light (6 mW/cm^2, λ_{max} = 470 nm) at 37 °C for 1, 3, 6, 9, 18, and 24 h. The monomer conversion during the reaction was monitored using ^{1}H NMR (D_2O), and the M_w and polydispersity index (PDI) were analyzed using SEC (Entry 1-6 in Table S1). We also confirmed that improved conversion and PDI in the condition without oxygen (Entry 7 in Table S1), while the addition of ascorbic acid as antioxidant resulted in increased conversion and PDI (Entry 8-9 in Table S1). We observed the complete photobleaching of calcein under the light irradiation for 24 h (Figure S1). The versatility of the system was further demonstrated using hydroxyethyl acrylate (HEA) with different polymerization property (Table S2).

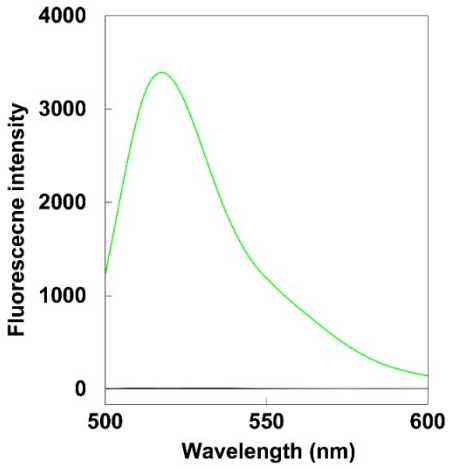

Figure S1. Fluorescence spectra of calcein before (green) and after (gray) light irradiation. The Calcein solution (100 nM, 1 mL) in the vial was incubated at 37°C for 24 hours under blue light (470 nm, 6 mW/cm²) irradiation. Subsequently, fluorescence measurements (Ex=490 nm) were performed on the solution as is.

Table S1. Summary of enzymes, monomer conversions, molecular weights, and PDIs in enzyme-gated PET-RAFT polymerizations conducted for 24 h.

Entry	Enzyme	Photocatalyst	Chain transfer agent	Ascorbic acid	Conversion (%)	Molecular weight (g/mol)	PDI
1	Carboxylesterase ($1\ gL^{-1}$)	CAM (250 μM)	CPADB (2.5 mM)	0	62	2.33×10^4	1.52
2	-	CAM (250 μM)	CPADB (2.5 mM)	0	0	-	-
3	Carboxylesterase ($1\ gL^{-1}$)	CAM (250 μM)	CPADB (1.25 mM)	0	42	3.12×10^4	1.71
4	Carboxylesterase ($1\ gL^{-1}$)	CAM (250 μM)	CPADB (5 mM)	0	69	1.64×10^4	1.39
5	Carboxylesterase ($1\ gL^{-1}$)	CAM (125 μM)	CPADB (2.5 mM)	0	55	2.47×10^4	1.54
6	Carboxylesterase ($1\ gL^{-1}$)	CAM (500 μM)	CPADB (2.5 mM)	0	70	2.02×10^4	1.48
7	-	Calcein (250 μM)	CPADB (5 mM)	0	71	2.72×10^4	1.41
8	Carboxylesterase ($1\ gL^{-1}$)	CAM (250 μM)	CPADB (5 mM)	2.5 mM	60	2.59×10^4	1.82
9	Carboxylesterase ($1\ gL^{-1}$)	CAM (250 μM)	CPADB (5 mM)	12.5 mM	67	2.06×10^4	2.08
10	Carboxylesterase ($0.1\ gL^{-1}$)	CAM (250 μM)	CPADB (2.5 mM)	0	55	2.51×10^4	1.60-
11	β-D-Galactosidase ($0.1\ gL^{-1}$)	CAM (250 μM)	CPADB (2.5 mM)	0	3	-	-
12	β-Glucosidase ($0.1\ gL^{-1}$)	CAM (250 μM)	CPADB (2.5 mM)	0	2	-	-
13	Alkaline phosphatase ($0.1\ gL^{-1}$)	CAM (250 μM)	CPADB (2.5 mM)	0		-	-
14	β-D-Galactosidase ($5\ gL^{-1}$)	FDGal (250 μM)	CPADB (2.5 mM)	0	52	3.16×10^4	1.74
15	-	FDGal (250 μM)	CPADB (2.5 mM)	0	2	-	-
16	β-Glucosidase ($10\ gL^{-1}$)	FDGlu (250 μM)	CPADB (2.5 mM)	0	61	2.77×10^4	1.69
17	-	FDGlu (250 μM)	CPADB (2.5 mM)	0	2	-	-
18	Alkaline phosphatase ($10\ gL^{-1}$)	FDP (100 μM)	CPADB (2.5 mM)	0	42	3.02×10^4	1.91
19	-	FDP (100 μM)	CPADB (2.5 mM)	0	2	-	-

Table S2. Summary of monomer conversions, molecular weights, and PDIs for various monomers in carboxylesterase-gated PET-RAFT polymerizations conducted for 24 h.

Entry	Monomer	Enzyme	Photocatalyst	Chain transfer agent	Conversion (%)	Molecular weight (g/mol)	PDI
20	HEA (500 mM)	Carboxylesterase (1 gL^{-1})	CAM (250 μM)	CPADB (2.5 mM)	21	2.7×10^4	1.45
21	HEA (500 mM)	-	CAM (250 μM)	CPADB (2.5 mM)	0	-	-

4. General procedure for light-controlled reaction of carboxylesterase-responsive PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by calcein AM.

A reaction solution consisting of PBS (990 μL), DMSO (10 μL), calcein AM (0.25 mg, 2.5×10^{-4} mmol), and carboxylesterase (1 mg) was prepared in a 9 mL glass vial. The vial cap was screwed on lightly, and the vial was left in the incubator at 37 °C for 3 h. Three hours later, HPMA (71.6 mg, 0.50 mmol) and CPADB (0.70 mg, 2.5×10^{-3} mmol) were added to the reaction solution, and the solution was irradiated with a blue LED light (6 mW/cm^2, λ_{max} = 470 nm), switching the light on and off every hour. The monomer conversion during the reaction was monitored using ^1H NMR (D_2O), and the M_w and PDI were analyzed by SEC (Figure S2).

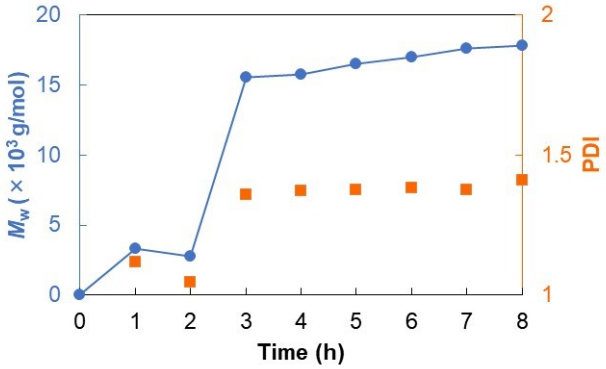

Figure S2. Molecular weight and PDI as a function of time in temporal controlled carboxylesterase-responsive PET-RAFT.

5. General procedure for light-controlled PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by calcein AM upon *in situ* addition of carboxylesterase.

A solution consisting of PBS (990 μL), DMSO (10 μL), calcein AM (0.25 mg, 2.5×10^{-4} mmol), HPMA (71.6 mg, 0.50 mmol), and CPADB (0.70 mg, 2.5×10^{-3} mmol) was prepared in a 9 mL glass vial. The vial cap was screwed on lightly, and the vial was left in an incubator at 37 °C and irradiated with blue LED light (6 mW/cm^2, λ_{max} = 470 nm) at 37 °C for 1 h. After 1 h, carboxylesterase (1 mg) was added. The monomer conversion during the reaction was analyzed using ^{1}H NMR (D_2O), and the M_w and PDI were analyzed using SEC (Figure S3).

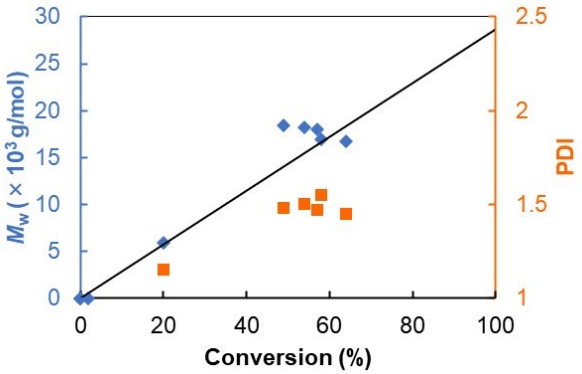

Figure S3. Molecular weight and PDI as a function of monomer conversion in carboxylesterase-responsive PET-RAFT initiated by *in situ* addition of carboxylesterase.

6. General procedure for studies on carboxylesterase selectivity in enzyme-responsive PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by calcein AM.

A solution consisting of PBS (990 μL), DMSO (10 μL), calcein AM (0.25 mg, 2.5×10^{-4} mmol) and either carboxylesterase (0.1 mg), β-D-galactosidase (0.1 mg),β-glucosidase (0.1 mg) or alkaline phosphatase (0.1 mg) was prepared in a 9 mL glass vial. The vial cap was screwed on lightly, and the vial was left in the incubator at 37 °C for 3 h. Three hours later, HPMA (71.6 mg, 0.50 mmol) and CPADB (0.70 mg, 2.5×10^{-3} mmol) were added to the vial, and the solution was irradiated with blue LED light (6 mW/cm^2, λ_{max} = 470 nm) at 37 °C for 24 h. After the reaction, the monomer conversion was determined using ^{1}H NMR (D_2O) (Entry 10-13 in Table S1).

7. General procedure for enzyme-responsive PET-RAFT polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) mediated by various enzyme-responsive photocatalysts and corresponding enzymes.

In a 9 mL glass vial, PBS (990 μL) and DMSO (10 μL) were added to each of the following formulations: calcein AM (0.25 mg, 2.5×10^{-4} mmol) and carboxylesterase (1 mg), fluorescein diphosphate tetraammonium salt (0.056 mg, 1.0×10^{-4} mmol) and alkaline phosphatase (10 mg), fluorescein di-β-D-galactopyranoside (0.16 mg, 2.5×10^{-4} mmol) and β-D-galactosidase (5 mg), and fluorescein di-β-D-glucopyranoside (0.064 mg, 1.0×10^{-4} mmol) and β-glucosidase (10 mg). The vial cap was screwed lightly, and the vial was left in the incubator at 37 °C for 3 h. Three hours later, HPMA (71.6 mg, 0.50 mmol) and CPADB (0.70 mg, 2.5×10^{-3} mmol) were added to the vial, and the solution was irradiated under a blue LED light (6 mW/cm^2, λ_{max} = 470 nm) at 37 °C for 24 h. After the reaction, the monomer conversions were determined using ^{1}H NMR (D_2O), and the M_w and PDI were analyzed using SEC (Entry 14-19 in Table S1).

8. General procedure for hydrogel synthesis via PET-RAFT co-polymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) and *N*,*N*'-methylenebismethacrylamide (mBIS) mediated by various enzyme-responsive photocatalysts and corresponding enzymes.

In a 9 mL glass vial, PBS (900 μL) and DMSO (100 μL) were added to each of the following formulations: fluorescein diphosphate tetraammonium salt (0.056 mg, 1.0×10^{-4} mmol) and alkaline phosphatase (10 mg), fluorescein di-β-D-galactopyranoside (0.16 mg, 2.5×10^{-4} mmol) and β-D-galactosidase (5 mg), and fluorescein di-β-D-glucopyranoside (0.064 mg, 1.0×10^{-4} mmol) and β-glucosidase (10 mg). The vial cap was screwed on lightly, and the vial was left in the incubator at 37 °C for 3 h. Three hours later, HPMA (286.4 mg, 2.0 mmol), CPADB (0.70 mg, 2.5×10^{-3} mmol) and mBIS (18.2 mg, 0.10 mmol) were added to the vial, and the solution was irradiated under a blue LED light (6 mW/cm^2, λ_{max} = 470 nm) at 37 °C for 1.5, 3, 5, and 24 h. At each time point, the inverted vials were photographed to monitor the gelation progress (Figure S4). Monomer conversions are shown in Table S3.

Table S3. Monomer conversion in hydrogel synthesis by enzyme-gated PET-RAFT polymerizations.

Entry	Enzyme	Photocatalyst	Chain transfer agent	Conversion (%)
22	Carboxylesterase ($1\ gL^{-1}$)	CAM (250 μM)	CPADB (2.5 mM)	95
23	β-D-Galactosidase ($5\ gL^{-1}$)	FDGal (250 μM)	CPADB (2.5 mM)	88
24	β-Glucosidase ($10\ gL^{-1}$)	FDGlu (250 μM)	CPADB (2.5 mM)	97
25	Alkaline phosphatase ($10\ gL^{-1}$)	FDP (100 μM)	CPADB (2.5 mM)	91

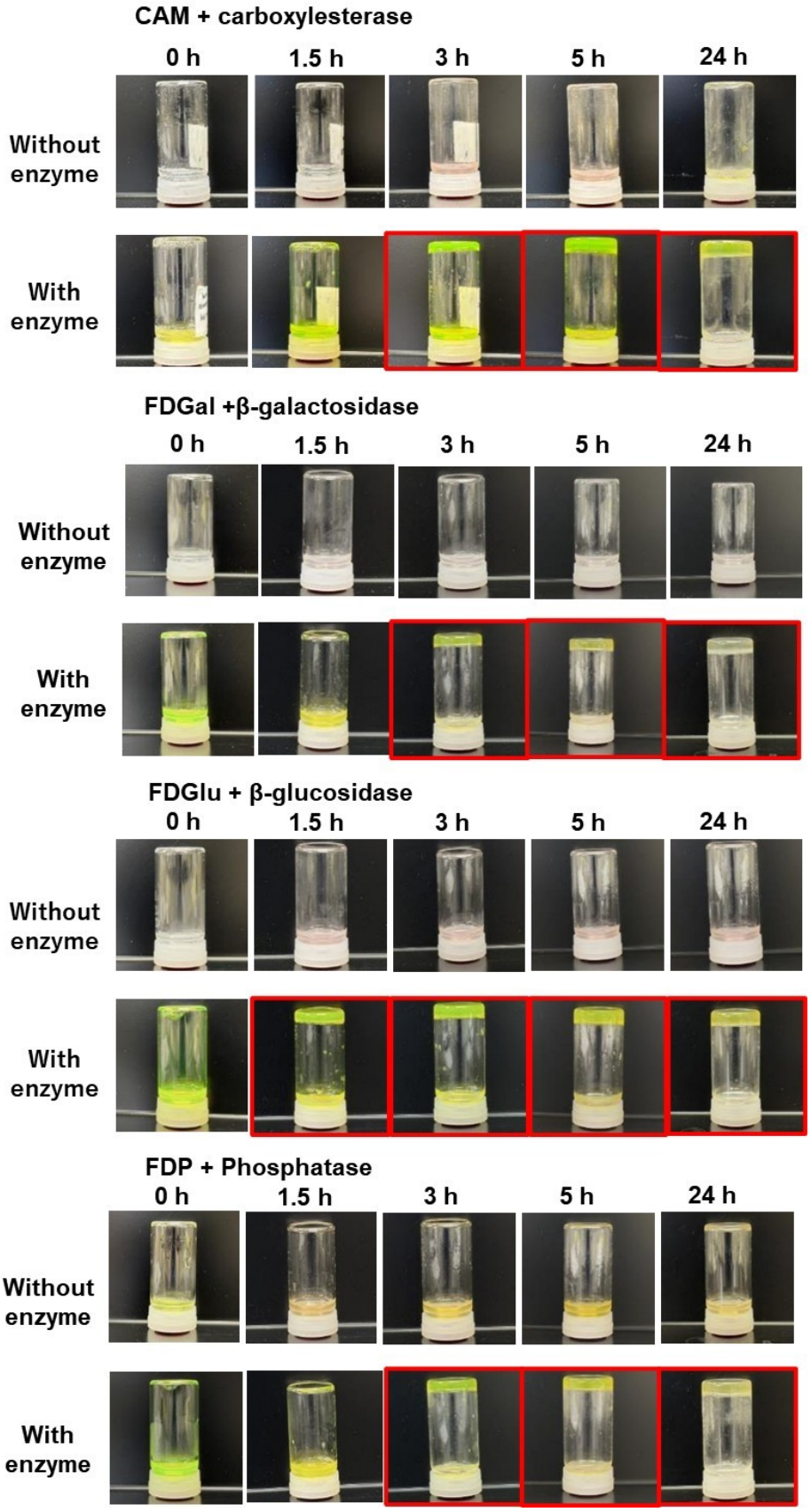

Figure S4. Hydrogel synthesis by enzyme-gated PET-RAFT. Obvious gelation was observed at the time points highlighted in red.