Supporting Information

Enzymatic Synthesis of Semi-IPNs within Hydrogel-Based Microfluidics

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Contents

1 Experimental section

1.1 Materials

N-isopropylacrylamide (NiPAAm, ≥99%), *N*,*N*-methylenebisacrylamide (BIS, 99%), lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, ≥95%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), acetylacetone (ACAC, ≥99%), maleimide (99%), cystamine hydrochloride (≥98%), 2,2'-dipyridyl disulphide (98%), phosphate buffered saline tablet (PBS), *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, ≥99%), *N*-hydroxysuccinimide (NHS, 98%), 2-(*N*-morpholino)ethanesulfonic acid (MES, ≥99%), sodium peroxodisulfate (99%), horseradish peroxidase (HRP, ≥250 U mg^{-1}), hydrogen peroxide (H_2O_2, 35%) were purchased from Sigma-Aldrich (Damstadt, Germany). *N,N'*-Bis(acryloyl)cystamine (BAC, 98%) was purchased from Alfa Aesar (Kandel, Germany). Sodium bicarbonate (99.5%) was purchased from ACROS Organics (Geel, Belgium). Sodium carbonate anhydrous (≥99.5%) was purchased from Honeywell Fluka (Seelze, Germany). AF488 NHS ester was purchased from Lumiprobe (Hannover, Germany). Acryloxyethyl thiocarbamoyl rhodamine B (acrylate-RhB) was purchased from Polysciences Company (Warrington, PA, USA). Silicone elastomer kit (PDMS) was purchased from DOW Corning (Midland, MI, USA). Deionized water was used for all experiments. All chemicals were used as received without further purification.

1.2 Instruments

Proton Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H NMR spectra were recorded on the Bruker Advance III spectrometer (Bruker Biospin, Germany) at 500 MHz. Chemical shifts of ^{1}H NMR were referred to TMS (δ = 0). The spectra were obtained using D_2O, DMSO-d_6 or chloroform-d as a solvent at 30 °C. All spectra were calibrated to the residual signal of the deuterated solvent. In the evaluation, the ppm values for the chemical shift δ were rounded to two decimal places and the values of the coupling constant J (in Hz) to one decimal place. The number of hydrogen atoms determined by integration is also given. The signal of the solvent served as the internal standard.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)

The MALDI-TOF mass spectra were measured in linear positive detection mode by an autoflex® speed MALDI-TOF system (Bruker Daltonics GmbH, Germany) equipped with a smartbeam™ II (modified Nd:YAG) laser having a wavelength of 355 nm. Sinapidic acid and α-cyano-4-hydroxycinnamic acid (HCCA) (both by Sigma Aldrich) were used as a matrix (both 10 g L^{-1}) dissolved in methanol and mixed at a ratio of 1:1 (v/v). 1 µL of a 0.5 g L^{-1} methanolic sodium trifluoroacetate solution was added per 100 µL matrix solution. The sample was prepared in deionized water at a concentration of 2 g L^{-1}, mixed with the matrix solution in a ratio of 1:1 (v/v) and spotted on the MALDI plate via the dried droplet method.[1] BSA of different batches was used for calibration and for verification. The samples were measured with an acceleration voltage of 19.5 kV, a laser attenuation of 30%, a laser repetition rate of 1 kHz, and a detector gain of 70x (3.446 kV). Each mass spectrum was recorded by accumulation of 8000 shots.

Confocal Laser Scanning Microscopy (CLSM)

The tracking of enzyme capture and enzyme/polymer release were performed on a Leica TCS SP8 AOBS (Leica, Germany) confocal microscope with excitation by diode pumped solid state (DPSS) lasers. The selection of laser wavelength was determined by the dye to be traced (λ_{ex} of 488 nm for AF488, λ_{ex} of 552 nm for RhB). Images were taken with a 10-fold magnification objective (dry). Acquired images were analyzed using ImageJ software.

Syringe Pump

For microfluidic experiments, a syringe pump LA-100, RS232 (Landgraf Laborsysteme, Langenhagen, Germany) and single-use plastic syringes (6 mL) were used. Variable dispensing volumes were programmed according to the test requirements.

Size Exclusion Chromatography (SEC)

SEC measurements were performed on SECcurity2 (PSS), equipped with GRAM Precolumn, Gram 30 Å, Gram 3000 Å columns under the following conditions: eluent-DMAc (5 g L^{-1} LiBr and 1% H_2O) at 50 °C, the injection volume was adjusted to 100 µL and the selected flow rate to 1 mL min^{-1} with a refractive index (RI) detection. The PNiPAAm measurement used the following conditions: concentration of the sample was ca. 4 mg mL^{-1} with a column of PolarGel-M (300 × 7.5 mm), eluent-DMAc (3 g L^{-1} LiCl) at 25 °C, the injection volume was adjusted to ca. 25 µL and the selected flow rate to 1 mL min^{-1} with a refractive index (RI) detection.

Fluorescence Spectroscopy

Fluorescence spectra for quantification of captured HRP were collected using a FS5 fluorescence spectrophotometer (Edinburgh Instruments Ltd, UK) equipped with a 150 W xenon lamp. The main measurements conditions for dye AF488 modified on HRP: λ_{ex} = 499 nm, λ_{em} = 520 nm, bandwidth = 2 nm.

1.3 Synthesis of HRP-AF488

HRP (20 mg, 0.456 μmol, 1 eq.) was dissolved in 50 mM carbonate buffer (4 mL, pH 8) and stirred for 30 min before adding AF488 NHS ester (1.0 mg, 1.37 μmol, 3 eq.) dissolved in 0.2 mL DMSO. After 20 h of stirring at room temperature, the mixture was extensively dialyzed against 1 mM PBS buffer (pH 7.4) for 3 days to remove all unbound AF488 NHS ester. All processes were performed under light protection. Finally, the purified mixture was freeze-dried overnight to get the product HRP-AF488, which was stored at -20 °C. Molecular weight was confirmed by MALDI-TOF-MS (M_{HRP}: 43100 Da and $M_{HRP\text{-}AF488}$: 43600 Da).

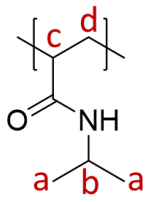
1.4 Synthesis of HRP-AF488-PDA

PDA was produced following the published procedure.[2, 3] HRP-AF488 (15 mg, 0.34 μmol, 1 eq.), NHS (2.0 mg, 17 μmol, 50 eq.) and EDAC (3.26 mg, 17 μmol, 50 eq.) were dissolved in 50 mM MES buffer (4 mL, pH 6.5) and stirred for 30 min before adding dropwise PDA (3.0 mg, 13.6 μmol, 40 eq.) dissolved in 50 mM MES buffer (0.5 mL, pH 6.5). After 20 h of stirring at room temperature, the mixture was extensively dialyzed against 1 mM PBS buffer (pH 7.4) for 3 days to remove all unbounded molecules. All processes were performed under light protection. Finally, the purified mixture was freeze-dried overnight to get the product HRP-AF488-PDA, which was stored at -20 °C. Molecular weight was confirmed by MALDI-TOF-MS ($M_{BSA\text{-}RhB\text{-}PDA}$: 43700 Da).

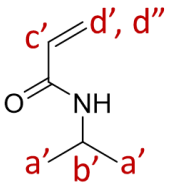
1.5 In-vial HRP-Promoted Radical Polymerisation

Free radical polymerisation of NiPAAm initiated by free HRP in vials was based on previously published work and performed at room temperature.[4] NiPAAm (67.9 mg, 0.6 mmol, 1 eq.), ACAC (3.0 mg, 0.03 mmol, 0.05 eq.), and HRP (0.264 mg, 0.006 μmol, 10^{-5} eq.) were dissolved in deionized water (3.27 mL, pH 7.0) and stirred for 5 min. Argon bubbling was applied to remove oxygen for 10 min before adding H_2O_2 (0.04 mg, 1.2 μmol, 0.002 eq.) for free radical polymerisation. The polymerisation was terminated by quenching the vial in liquid nitrogen.

The products prepared after different polymerisation times (5, 10, 15, 20, 40, 60 min) were directly lyophilized and tested by NMR, SEC, etc. without further purification. The ^{1}H NMR spectra of the monomer NiPAAm and the polymer PNiPAAm initiated by free HRP in vials are shown in Figure 2 and their chemical shifts are as follows:



^{1}H NMR (500 MHz, $CDCl_3$, δ): 3.78-4.13 (d, H_b), 1.93-2.57 (H_c), 1.49-1.83 (H_d), 1.03-1.25 (H_a).



^{1}H NMR (500 MHz, $CDCl_3$, δ): 6.27 (d, $^{3}J_{HH}$ = 17.13 Hz, 1H, $H_{c'}$), 6.05 (dd, $^{3}J_{HH}$ = 9.12 Hz, $^{4}J_{HH}$ = 6.84 Hz, 1H, $H_{d'}$), 5.60 (d, $^{3}J_{HH}$ = 10.28 Hz, 1H, $H_{d"}$), 4.15 (octet, $^{3}J_{HH}$ = 6.74 Hz, 1 H, $H_{b'}$), 1.18 (d, $^{3}J_{HH}$ = 6.36 Hz, 6H, $H_{a'}$).

Table S1. Compositions for free HRP initiated free radical polymerisation in vial.

	NiPAAm[a]	HRP	ACAC	H_2O_2
mol%	1	10^{-5}	0.05	0.002
Amount	67.9 mg, 0.6 mmol	0.264 mg, 0.006 µmol	3.0 mg, 0.03 mmol	0.04 mg, 1.2 µmol

[a] Concentration: 2 wt%.

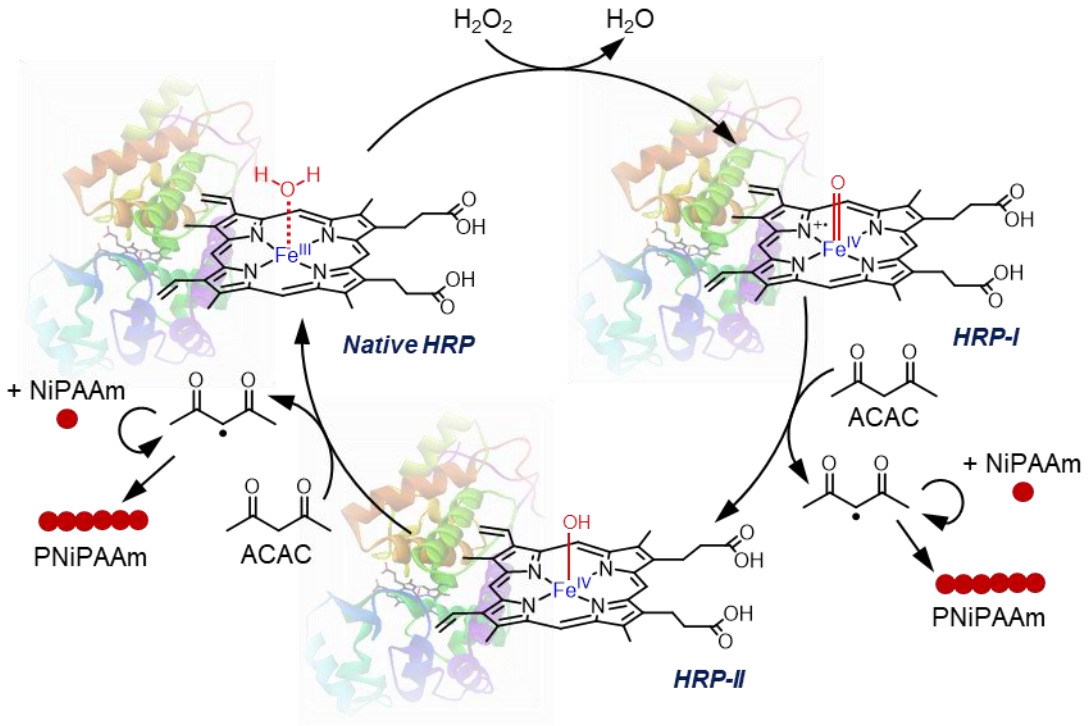
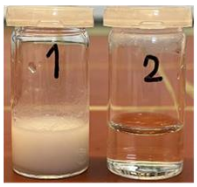


Figure S1. Simplified HRP catalytic cycle and recognized free radical formation mechanism in the process. Native HRP was first oxidized by hydrogen peroxide to obtain HRP-I. By abstracting two individual hydrogen atoms from the reducing substrate (ACAC), HRP-I was sequentially converted into HRP-II and native HRP, producing two free radical species that initiated the polymerisation. Modified from Ref.[4]

a



b

c

Sample	NiPAAm	ACAC	HRP	H_2O_2	Ar bubbling
1	Yes	Yes	Yes	Yes	Yes
2	Yes	Yes	Yes	Yes	No
3	Yes	Yes	No	Yes	Yes
4	Yes	Yes	Yes	No	Yes

Figure S2. Free radical polymerisation of NiPAAm initiated by free HRP in vials. The photos of the solution under a hot water bath at the reaction time of (a) 1 min and (b) 60 min. (c) The compositions and argon bubbling condition of each sample, which are consist with the ones shown in (a) and (b).

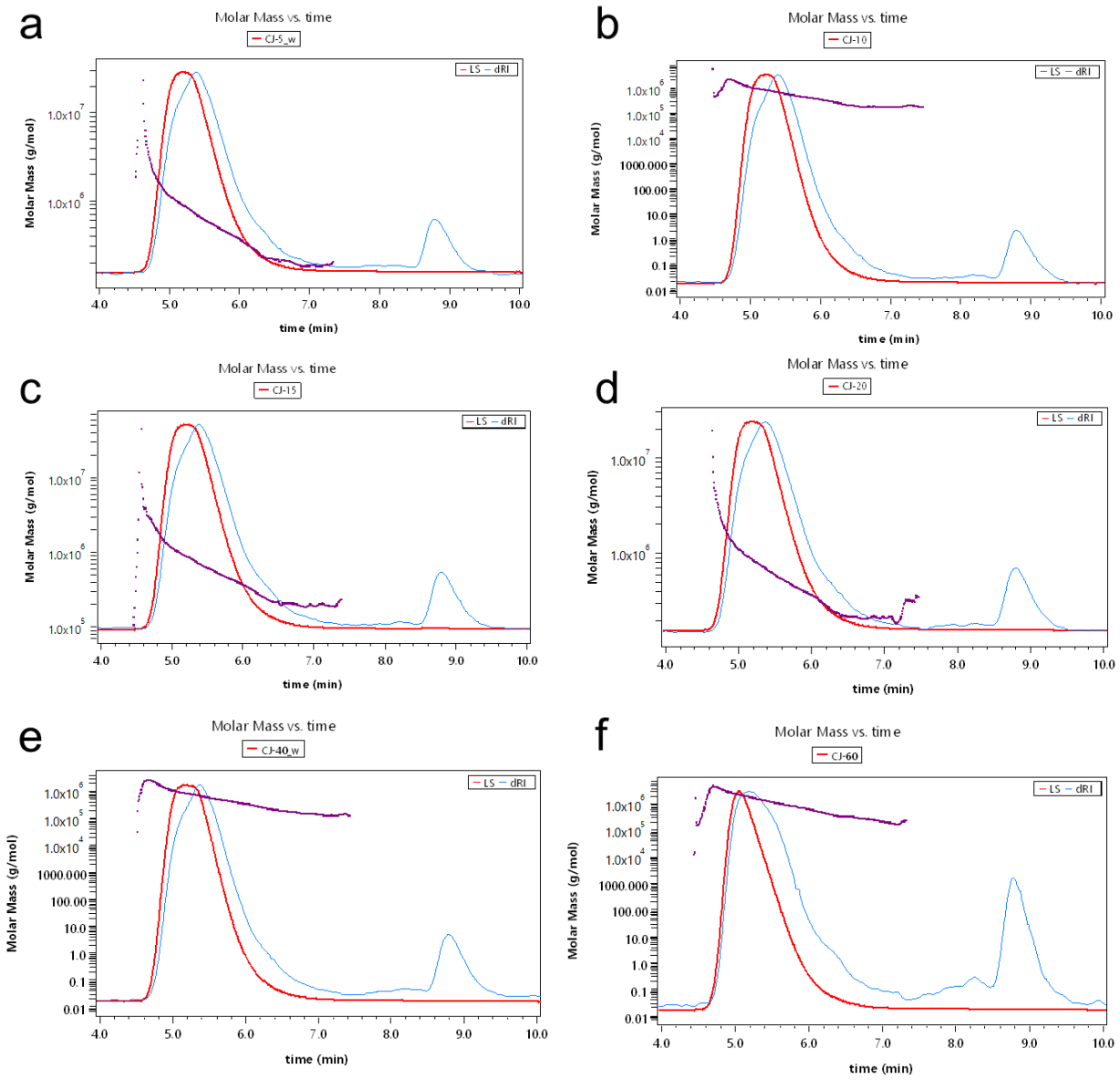


Figure S3. SEC chromatograms of formed PNiPAAm with the polymerisation time of (a) 5 min, (b) 10 min, (c) 15 min, (d) 20 min, (e) 40 min, (f) 60 min under SEC measurements. The blue and red curves refer to refractive index (RI) and light scattering (LS) chromatograms, respectively. The purple curves refer to the evolution of molar mass with retention time detected by MALLS detector.

Table S2. Molar mass characteristics of PNiPAAm initiated by free HRP in vials.

Polymerisation Time (min)	Concentration (mg mL^{-1})	M_n[a] (g mol^{-1})	M_w[a] (g mol^{-1})	Đ[a] (M_w/M_n)
5	3.82	522,800	690,900	1.32
10	3.82	487,700	651,100	1.33
15	4.00	535,400	693,800	1.30
20	3.69	525,600	676,800	1.29
40	3.90	486,500	660,500	1.36
60	3.95	490,500	667,500	1.36

[a] Measured by SEC at 25 °C with a PolarGel-M column (300 × 7.5 mm), using HPLC-Pump 1200 pump system (Agilent technologies) equipped with a multi angle laser light scattering (MALLS) detector (MiniDAWN TREOS II, Wyatt Technology) and a RI detector (K-2301 from KNAUER). DMAc with 3 g L^{-1} LiCl was used as an eluent and the flow rate was 1 mL min^{-1}.

1.6 Preparation of PNiPAAm-BAC-BIS µHDs Array by Photopolymerisation

For the preparation of hydrogel precursor solution, the cross-linker BAC was first dissolved in ethanol and stirred for 20 min, then the monomer NiPAAm (930.8 mg, 12.5 wt%) and the other cross-linker BIS dissolved in deionized water were added. The entire amount of cross-linker is fixed at 2 mol% and with molar ratios (BAC:BIS) of 2:1. 0.71 mL ethanol was used to dissolve all the BAC and the other solvent was 5.90 mL deionized water. The amount of photoinitiator is 0.65 mol% to the monomer, which was added under light protection. The precursor solutions were purged with argon for 15 min to remove oxygen. Before the photopolymerisation of hydrogel arrays, the Menzel glass slide (76 mm × 26 mm × 1 mm) was cleaned with isopropanol, MilliQ, and ethanol sequentially in an ultrasonic bath.

The preparation of the µHDs arrays was divided into five steps and derived from a previously published procedure.[3, 5, 6] Firstly, the hydrogel precursor solution was transferred to the reaction chamber of the POM mould. Then, the washed glass slide was aligned on the chamber and the patterned photomask was aligned on the glass slide. According to the design principle, an even layer without any trapped air was formed between the glass slide and the reaction chamber of the POM mould. Afterwards, the reaction chamber was exposed to a UV lamp (DELOLUX 04,

DELO, Windach, Germany) with a light power of 8 mW cm^{-2} on the sample surface. The emission spectrum for the photopolymerisation ranges from 315 to 500 nm and the irradiation time is 7 s. Next, the glass slide with the covalently attached cylinder-shaped µHDs was separated from the POM mould and immersed in the deionized water overnight to remove all the unbound precursor solutions. After the as-prepared µHDs reached equilibrium in deionized water, dried and then wiped the glass slide with isopropanol without touching the µHDs. Finally, the PDMS-on-glass chip was sealed by aligning the glass slide on the PDMS sheet. The µHDs used for control experiments were prepared in the same way, except that BAC was removed.

1.7 Microfluidic Setup

Preparation of Photomasks

Photomask for patterning (array with diameter 350 µm) was designed with the CAD software Autodesk Inventor and produced on a black and white flat film by photo plotting (MIVA 26100 ReSolution, MIVA Technologies, Schönaich, Germany). Based on previously published procedures [3, 5-7]

Preparation of PDMS Sheets

The PDMS sheet used to construct the PDMS-on-glass microfluidic devices was manufactured by hard and soft lithography in a standard process. This step was based on previously published procedures. [3, 5-7] The mould of the PDMS sheet was prepared by the following method. First, a glass substrate was rinsed with acetone, isopropanol, and deionized water. Afterwards, three resist layers (DFR, 50µm) were laminated on the glass slide and baking at 85 °C for 3 min was performed after each layer. Next, the resist was irradiated with UV light through a photomask for 90 s, and baked at 85 °C for 40 min. Afterwards, the resist was evolved in a developer and rinser bath and finally baked at 85 °C for 1 h. The manufacturing accuracy, i.e., the heights of the chambers, was investigated by confocal microscopy (µsurf explorer, Nano Focus, Germany). Subsequently, the PDMS elastomer base agent and the curing agent (Sylgard 184, Dow Corning) were mixed at a mass ratio of 10:1 (according to the supplier's instructions) and stirred for 15 min until distributed evenly. The mixture was then poured onto the PDMS master, degassed, and cured overnight at 40 °C. Finally, the PDMS sheet was peeled off from the mould and perforated to form the fluidic inlet and outlet by a biopsy punch (Kai Industries Co., Ltd,

Ø: 1.5 mm). Prior to assembling the microfluidic device, the PDMS sheet was cleaned by ultrasonic treatment in isopropanol.

Assembly of Microfluidic Setup

The whole microfluidic setup was composed of a syringe pump, a bubble trap, and a microfluidic device, which following the previous methods to be assembled.[3, 5] The glass slide with attached hydrogel arrays was rinsed with deionized water, and dried with a cotton swab. Note that the μHDs cannot be touched during drying because they are fragile and easily moved. Then, the prepared PDMS sheet was placed onto and aligned with the glass slide to form the microfluidic device (PDMS-on-glass chip), thereby sealing the chip. The chip was covered with an aluminium frame with a transparent acrylic glass window and placed in an aluminium clip to prevent the PDMS sheet from loosening from the glass slide during the test. Finally, the microfluidic pump (LA-100, LANDGRAF HLL, Germany), the bubble trap (LVF-KBT-L, Darwin Microfluidics, Paris, France), and the microfluidic device were connected in series through PTFE tubes and Cannulas (TechLab, Germany). The bubble trap was used to remove bubbles in the solution. Based on the test, the response time (that is, the time required to fill the microfluidic chip and reach the outlet tube) was about 8 min at a pumping flow rate of 5 μL min^{-1}.

1.8 HRP Capture by Disulphide Bonds in Microfluidics

In the HRP capture part, 0.01 M TCEP aqueous solution was first purged at the flow rate of 5 μL min^{-1} for 60 min, followed by deionized water washing for 30 min. Afterwards, 50 μM HRP-AF488-PDA aqueous solution was purged at the flow rate of 5 μL min^{-1} for 60 min, followed by 2 mM PBS buffer (pH 7.4) washing for 60 min. The flow rate of all the chip washing was 10 μL min^{-1}. The whole process was observed by confocal laser fluorescence microscope (CLSM, Leica SP5, Germany) under both laser field (λ_{ex}: 488 nm, laser intensity: 15%) and bright field. The fluorescence intensities were analyzed by ImageJ.

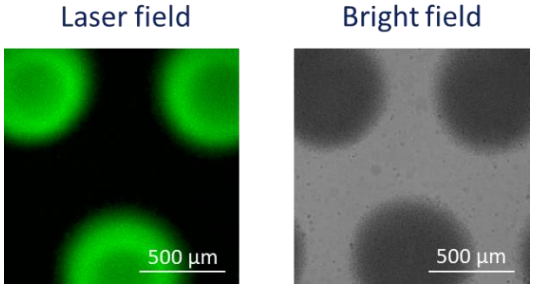


Figure S4. Fluorescence micrograph of HRP-captured PNiPAAm μHDs by disulphide bond formed between HRP-AF488-PDA and μHDs, and its corresponding brightfield micrograph.

Two controls were performed to verify the successful capturing of HRP. One was purging 50 μM HRP-AF488-PDA aqueous solution at the flow rate of 5 μL min^{-1} for 60 min, followed by 2 mM PBS buffer (pH 7.4) washing for 60 min. The other was consistent with the purging experiment of the HRP capture, except that HRP-AF488-PDA was replaced by HRP-AF488. The flow rate of all the chip washing was 10 μL min^{-1}.

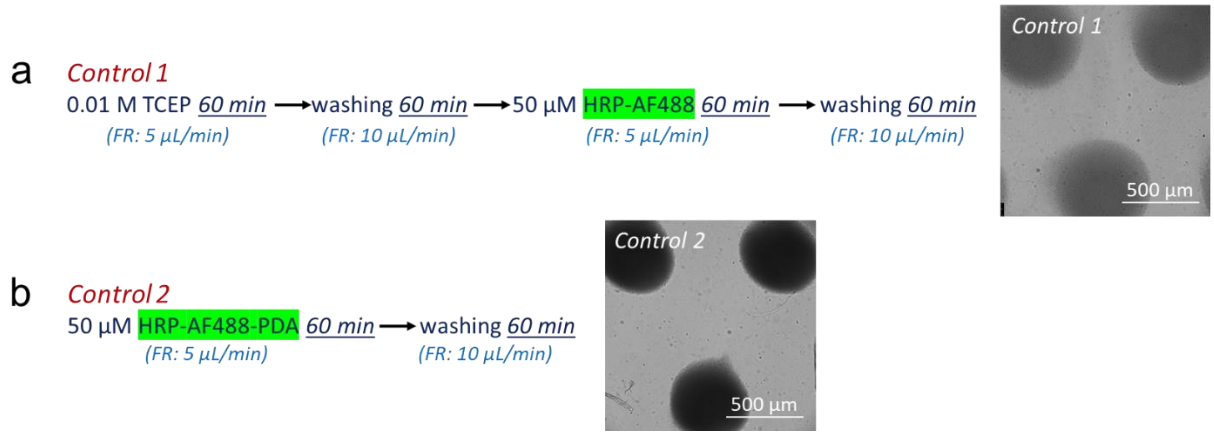


Figure S5. The perfusion order of two controlling strategies. The first control was conducted without PDA modified HRP and second control was conducted without reducing agent (TCEP) perfusion before the HRP capture, leading to no captured enzyme in the microfluidic device. Bright field micrographs correspond to each control.

1.9 Quantification of HRP Captured by μHDs in Microfluidics

The μHDs in the PDMS-on-chip chip were used to quantify the captured HRP. Dissociated the microfluidic chip after HRP capture. At this moment, μHDs were partially attached to the glass slide and partially attached to the surface of the PDMS microchannel. Immersed the PDMS sheet and glass slide (all μHDs should be immersed) in 12 ml of 0.03 M TECP aqueous solution, and arranged on the shaker for 10 h. Repeat the above operation three times to completely release the μHDs-captured enzyme, collect the rinsing solution for fluorescence measurements. After the time points, the samples were taken and analyzed using a fluorescence spectrophotometer (Edinburgh Instruments Ltd, UK, λ_{ex}: 499 nm and λ_{em}: 520nm) according to the calibration curves of HRP-AF488-PDA (Figure 5.1) in PBS buffer at pH 7.4 to quantify the captured HRP. The capturing efficiency was calculated from Equation (1):

$$\text{capturing efficiency} = \frac{\text{captured amount of HRP}}{\text{perfused amount of HRP}} \times 100\% \quad (1)$$

Since the same developed microreactor was used, the volume of the microfluidic chamber was known to be 26.88 μL.[3] This could be used to calculate the concentration of the captured hydrogel in the chip.

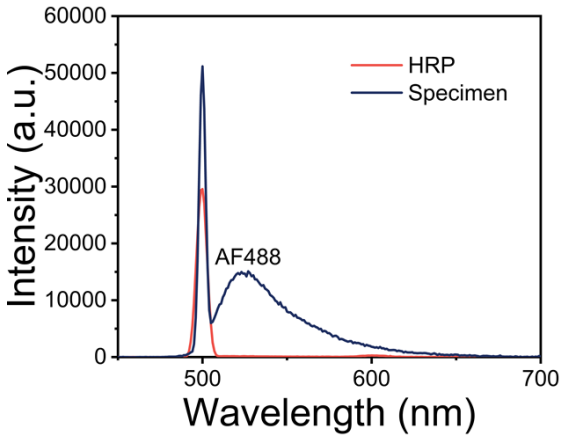


Figure S6. Fluorescence spectra for quantification of captured HRP-AF488-PDA released in excess TCEP solution and free HRP (λ_{ex} = 499 nm, λ_{em} = 520 nm).

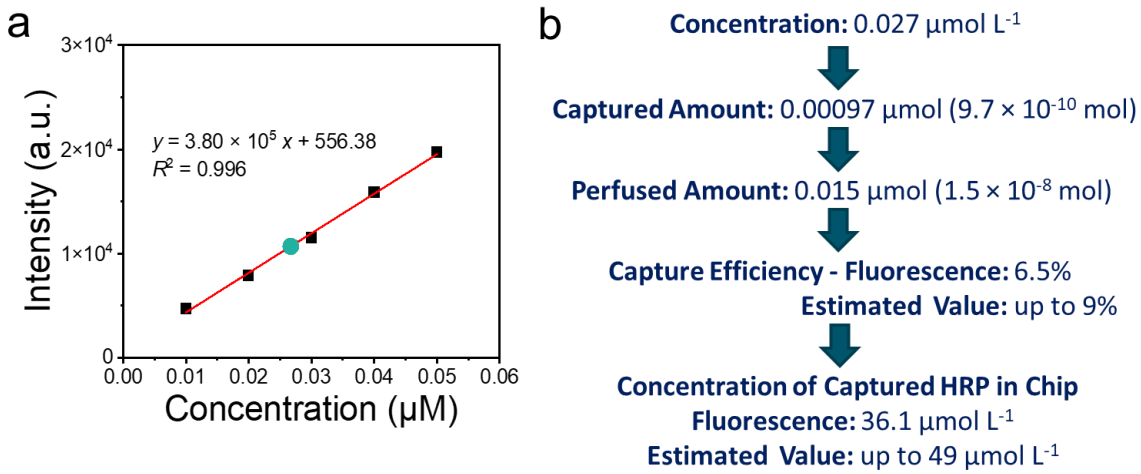


Figure S7. (a) Calibration curve of the AF488 labeled HRP in PBS pH 7.4. The measurement was carried out at λ_{ex}: 499 nm and λ_{em}: 520 nm using fluorescence spectroscopy. The green dot in the figure was the samples to be determined. (b) Calculation process of HRP-AF488-PDA capture efficiency and the concentration of captured HRP-AF488-PDA in the microfluidic chip.

1.10 HRP-Promoted Radical Polymerisation in Microfluidics

HRP-promoted radical polymerisation in the microfluidic chip was performed after HRP-AF488-PDA was captured by μHDs. After the last washing step in the HRP capture was complete, the monomer solution was passed through the chip. The test conditions of the monomer solution include different flow rates (3 μL min^{-1} and 5 μL min^{-1}), different monomer concentrations (C_{NiPAAm} of 0.18 M and 1 M), and different perfusion methods (static incubation condition and maleimide), etc. The polymerisation was terminated by quenching the effluent in liquid nitrogen.

The preparation of 0.18 M monomer solution was as follows: NiPAAm (67.9 mg, 0.6 mmol, 1 eq.) was dissolved in deionized water (3.27 mL, pH 7.0), which was simultaneously added by ACAC (3.0 mg, 0.03 mmol, 0.05 eq.) and H_2O_2 (0.04 mg, 1.2 μmol, 0.002 eq.) and stirred for 5 min. Argon bubbling was applied to remove oxygen for 10 min before perfusing it into the microfluidic chip for polymerisation. The preparation of 1.0 M monomer solution was as follows: NiPAAm (67.9 mg, 0.6 mmol, 1 eq.) was dissolved in deionized water (0.6 mL, pH 7.0), which was simultaneously added by ACAC (3.0 mg, 0.03 mmol, 0.05 eq.) and H_2O_2 (0.04 mg, 1.2 μmol, 0.002 eq.) and stirred for 5 min. Argon bubbling was applied to remove oxygen for 10 min before perfusing it into the microfluidic chip for polymerisation.

Table S3. Microfluidic measurements with different parameters of free radical polymerisation initiated by captured HRP-AF488-PDA on μHDs.

Measure-ments	Flow Rate	C_{NiPAAm}	Static Incubation in the Chip	Perfusate after Incubation	Maleimide treatment
I	5 μL min^{-1}	0.18 M	-	-	-
II	3 μL min^{-1}	0.18 M	-	-	-
III	5 μL min^{-1}	0.18 M	-	-	0.1 M for 1 h
IV	5 μL min^{-1}	1 M	-	-	-
V	5 μL min^{-1}	1 M	2 h	water	-
VI	5 μL min^{-1}	1 M	1 h	0.01 M TCEP	-

Figure S8. Photo of the effluent collected in a centrifuge tube in *Measurement I* after being heated in a hot water bath.

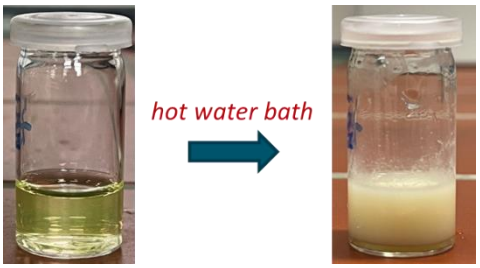


Figure S9. Free radical polymerisation of NiPAAm initiated by free HRP-AF488-PDA in vials. The photos of the reaction solution before and after the hot water bath after one-hour incubation, the green fluorescence came from the dye AF488 modified on HRP. All compositions were consistent with the *Measurement I*, except that free HRP was replaced by free HRP-AF488-PDA.

Table S4. Compositions of the monomer solutions for free radical polymerisation initiated by μHDs-captured HRP-AF488-PDA in microfluidics, where the acrylate-RhB was used to track polymer formation.

	NiPAAm[a]	Acrylate-RhB	ACAC	H_2O_2
mol%	1	2.5×10^{-5}	0.05	0.002
Amount	67.9 mg, 0.6 mmol	0.01 mg, 0.015 μmol	3.0 mg, 0.03 mmol	0.04 mg, 1.2 μmol

[a] Concentration: 2 wt%.

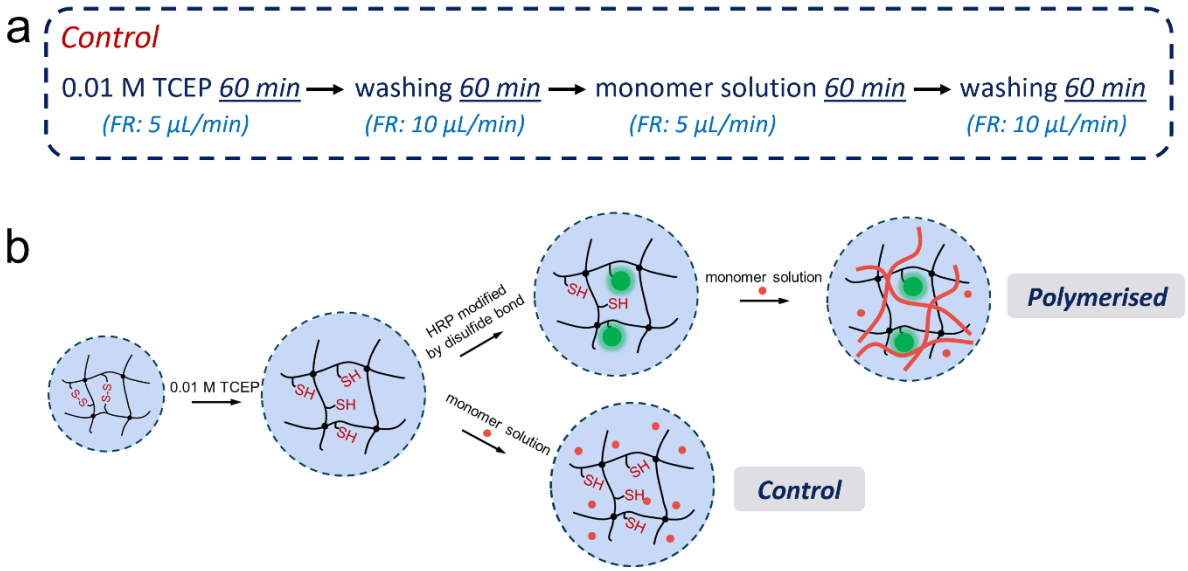


Figure S10. (a) The perfusion order and (b) schematic drawing of the controls for the polymerisation, which skipped the HRP-AF488-PDA capture process before monomer solution perfusion, leading to an incomplete initiation system. The monomer solution contained dye-modified monomer acrylate-RhB.

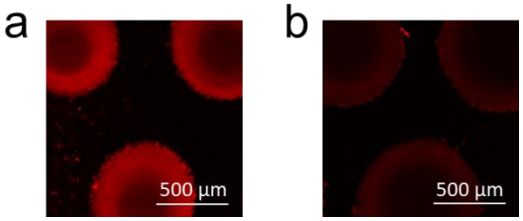


Figure S11. Fluorescence micrographs of the (a) interpenetrating and/or entangled polymers on µHDs (same as Figure 5c, shown here again for comparison) and (b) the control without polymer formation.

2 References

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