Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information for

Control of microenvironment around enzymes by hydrogels

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Materials

All the reagents and solvents were commercially available and used as received without further purification. Cytochrome P450 $_{SP\alpha}$(CYP152B1)[1, 2] was prepared according to our previous report.[3, 4]

Measurements

MALDI–TOF MS spectra were recorded in the linear positive mode on a Bruker autoflex speed mass spectrometer. Mass number was calibrated by four peptides, i.e., angiotensin II ($[M+H]^+$ 1046.5418), angiotensin I ($[M+H]^+$ 1296.6848), substance P ($[M+H]^+$ 1347.7354), and bombesin ($[M+H]^+$ 1619.8223). UV–vis spectra were recorded on a JASCO V-650 spectrometer.

Oxidation reaction mechanism by P450[5-7]

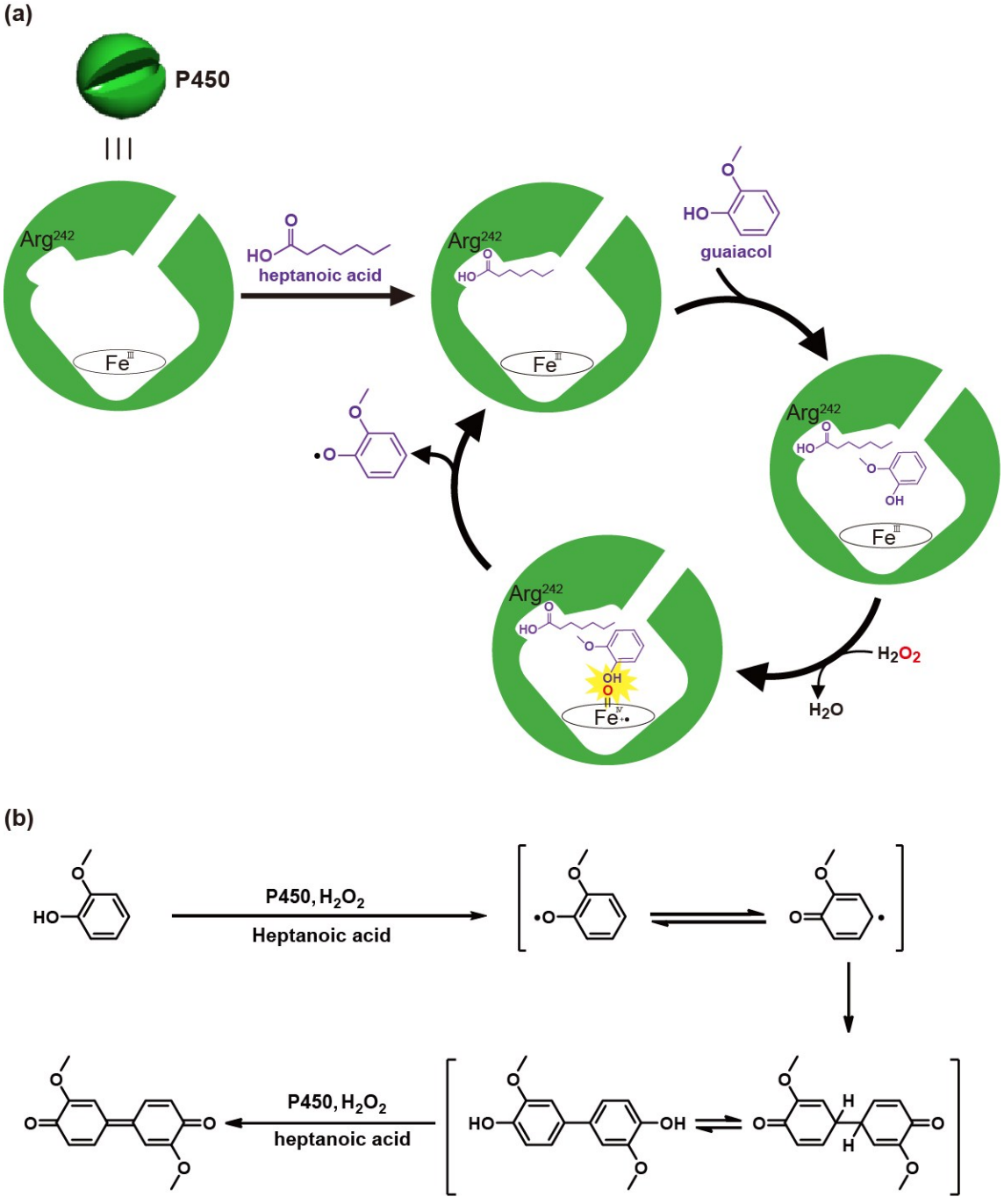
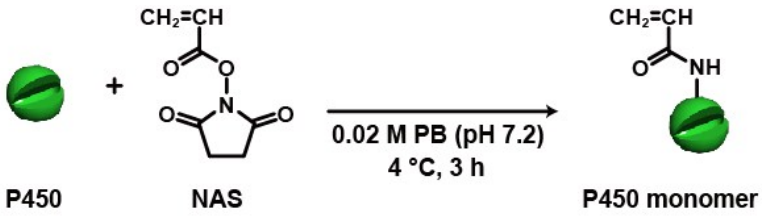


Figure S1. (**a**) Oxidation reaction mechanism of non-natural guaiacol (substrate) by P450 using heptanoic acid (decoy molecule). (b) The reaction mechanism of preparation of two-electron oxide of guaiacol dimer (monitor compound).

Preparation of P450-modified gels

Preparation of P450 monomer



Scheme S1. Preparation of P450 monomer.

N-Acryloxysuccinimide (NAS) was added to 16.6 μM P450 dissolved in 0.02 M phosphate buffer (PB) (pH 7.2), and the solution was stirred at 4 ºC for 3 h. To remove unreacted NAS and other low molecular compounds, we performed ultrafiltration using an Amicon® Ultra-4 (MWCO 10,000). In MALDI-TOF MS spectra of the upper layer of ultrafiltration, a peak with a higher molecular weight than molecular weight of P450 was observed (Fig. S1), thus preparation of P450 monomer was confirmed. The concentration of the P450 solution obtained was confirmed by UV-Vis measurement (1.0 mL, 3.2 μM).

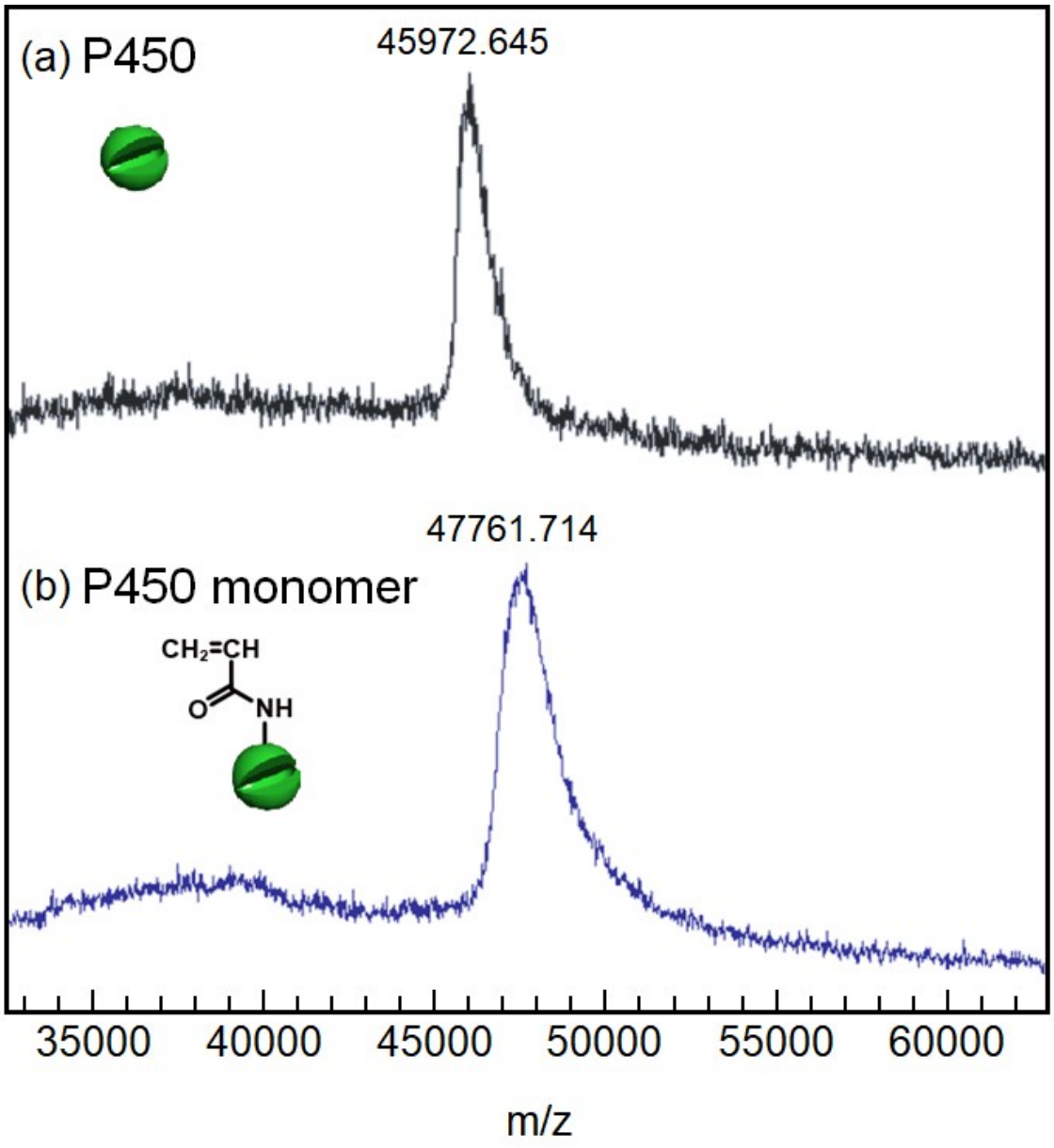
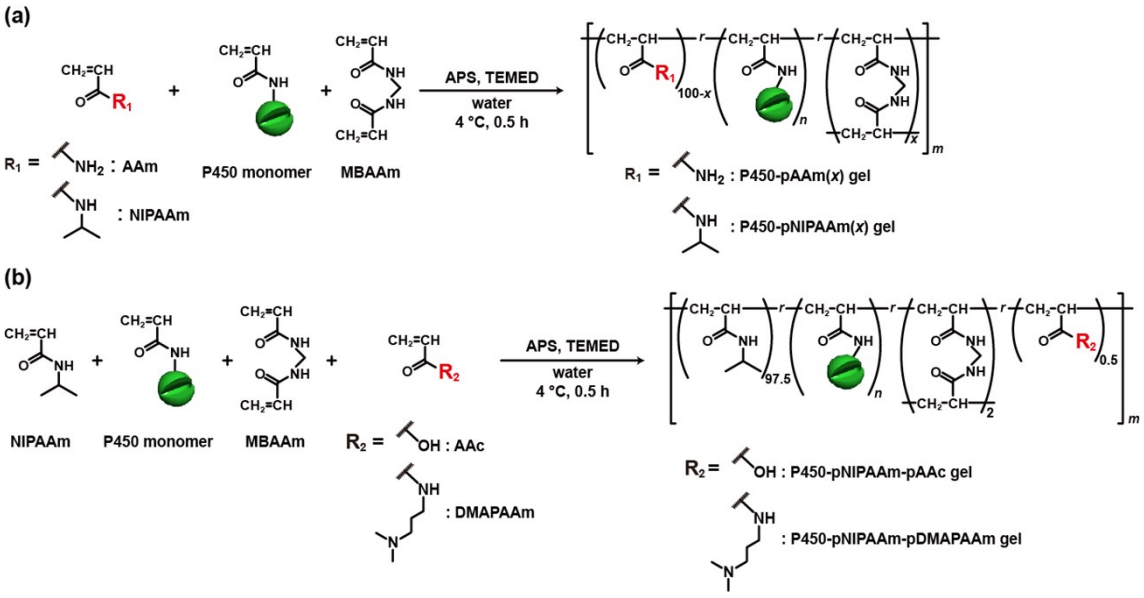


Figure S2. MALDI-TOF MS spectra of (a) P450 and (b) P450 monomer.

Preparation of P450 gels



Scheme S2. Preparation of (a) P450-pAAm(*x*) gel and P450-pNIPAAm(*x*) gel, (b) P450-pNIPAAm-pAAc gel and P450-pNIPAAm-pDMAPAAm gel.

Various monomers, such as acryl amide (AAm), *N*-isopropylacrylamide (NIPAAm), acrylic acid (AAc), or *N*,*N*-dimethylaminopropyl acrylamide (DMAPAAm), and *N*,*N*'-methylenebisacrylamide (MBAAm) was dissolved in water. Upon the addition of ammonium persulfate (APS) and *N*,*N*,*N*',*N*'-tetramethyl-1,2-ethanediamine (TEMED), the reaction mixture immediately became a gel (Tables S1-4). The gel was washed with water to remove the unreacted monomers and initiators and was subsequently soaked in an excess of 0.02 M PB (pH 7.2).

Table S1. Preparation of P450-pAAm(2) gel.

Water (mL)	P450 monomer (mg/nmol)	AAm (g/mmol)	MBAAm (mg/μmol)	APS (mg/μmol)	TEMED (mg/μmol)
2.0	0.32/6.7	0.26/3.9	12/80	10/46	4.7/40

Table S2. Preparation of P450-pNIPAAm(*x*) gels.

X	Water (mL)	P450 monomer (mg/nmol)	pNIPAAm (g/mmol)	MBAAm (mg/μmol)	APS (mg/μmol)	TEMED (mg/μmol)
0.5	2.0	0.32/6.7	0.45/4.0	3.1/20	10/46	4.7/40
1			0.45/4.0	6.2/40		
2			0.44/3.9	12/80		
5			0.43/3.8	31/200		
10			0.41/3.6	62/400		

Table S3. Preparation of P450-pNIPAAm-pAAc gel.

Water (mL)	P450 monomer (mg/nmol)	pNIPAAm (g/mmol)	AAc (mg/μmol)	MBAAm (mg/μmol)	APS (mg/μmol)	TEMED (mg/μmol)
2.0	0.32/6.7	0.44/3.9	1.4/20	12/80	10/46	4.7/40

Table S3. Preparation of P450-pNIPAAm-pDMAPAAm gel.

Water (mL)	P450 monomer (mg/nmol)	pNIPAAm (g/mmol)	DMAPAAm (mg/μmol)	MBAAm (mg/μmol)	APS (mg/μmol)	TEMED (mg/μmol)
2.0	0.32/6.7	0.44/3.9	3.1/20	12/80	10/46	4.7/40

P450 concentration in P450 gels

The concentration of P450 in the gel was calculated from Lambert-Beer's law using the result of UV-Vis measurement of hydrogel (Table S5). The gels were cut into 10 × 10 × 1 mm^3 and the absorption measured at 420 nm. Since the P450-pNIPAAm(10) gel was cloudy (Fig. S3), the concentration could not be calculated.

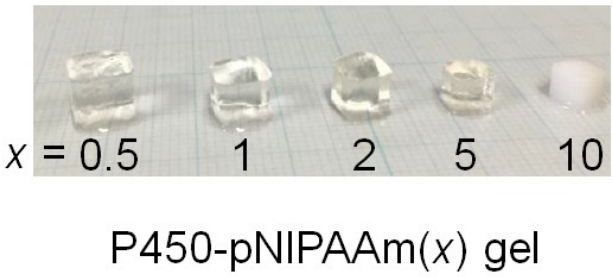


Figure S3. Photograph of P450-pNIPAAm(*x*) gel

Table S5. Introduction amount of P450 in the gels.

P450 gels	*n* (mol% in the P450 gel)
P450-pAAm(2) gel	1.5×10^{-4}
P450-pNIPAAm(0.5) gel	1.3×10^{-4}
P450-pNIPAAm(1) gel	1.3×10^{-4}
P450-pNIPAAm(2) gel	1.4×10^{-4}
P450-pNIPAAm(5) gel	1.3×10^{-4}
P450-pNIPAAm(10) gel	–
P450-pNIPAAm-pAAc gel	1.3×10^{-4}
P450-pNIPAAm-pDMAPAAm gel	1.3×10^{-4}

Catalytic activity of P450 gels

5 × 5 × 5 mm^3 (125 μL) of P450 gel was immersed in 5% EtOH in 0.02 M phosphate buffer (PB) (pH 7.2) (1.0 mL) containing guaiacol as substrate, heptanoic acid as decoy molecule, and 50 mM H_2O_2 at 26 4 ºC for 5 min. The P450 gel was picked up and immersed in $CHCl_3$ (500 μL) for 5 min to extract a product of enzymatic reaction. The concentration of the product in $CHCl_3$ was calculated from Lambert-Beer's law using the result of UV-Vis measurement.

$A_{470\ nm} = \varepsilon_{470\ nm} \times C_{produt} \times l$

$A_{470\ nm}$ (absorbance at 470 nm)

$\varepsilon_{470\ nm}$: $2.66 \times 10^4\ M^{-1}cm^{-1}$

C_{produt} (concentration of the product of enzymatic reaction)

l = 1 cm

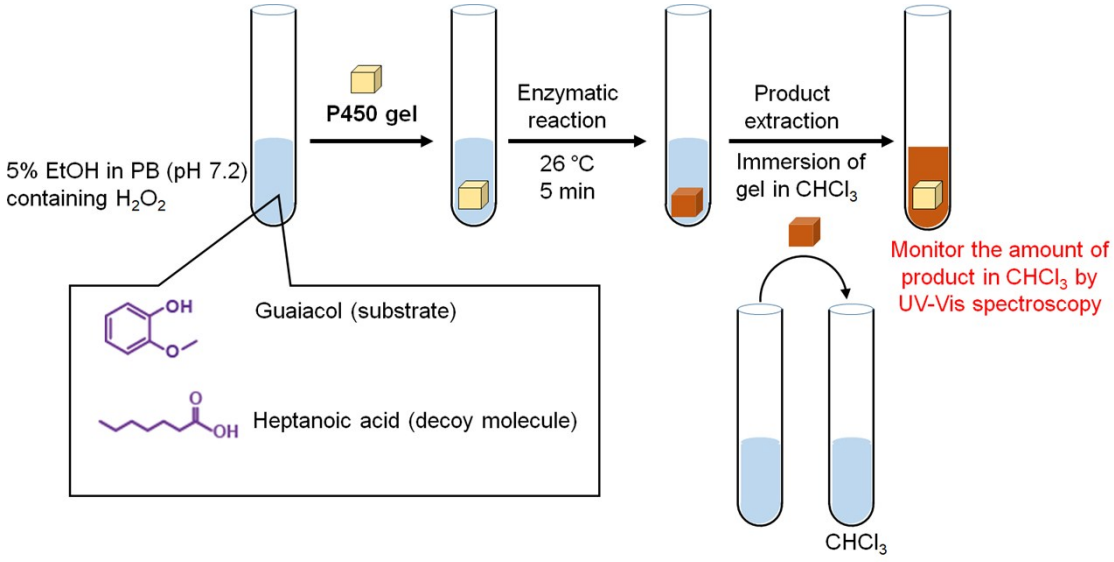
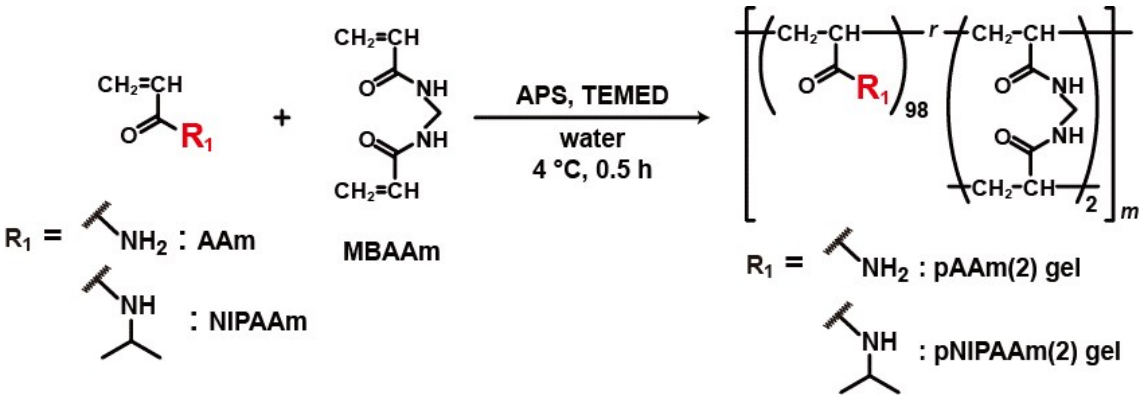


Figure S4. Experimental procedure of enzymatic reaction of P450 gel.

Incorporation of guaiacol into gels

Preparation of pAAm(2) gel and pNIPAAm(2) gel



Scheme S3. Preparation of pAAm(2) gel and pNIPAAm(2) gel.

AAm or NIPAAm and MBAAm was dissolved in water. Upon the addition of APS and TEMED, the reaction mixture immediately became a gel (Table S6). The gel was washed with water to remove the unreacted monomers and initiators and was subsequently soaked in an excess of 0.02 M PB (pH 7.2).

Table S6. Preparation of pAAm(2) gel and pNIPAAm(2) gel.

Hydrogels	Water (mL)	AAm (g/mmol)	NIPAAm (g/mmol)	MBAAm (mg/μmol)	APS (mg/μmol)	TEMED (mg/μmol)
pAAm(2) gel	2.0	0.26/3.9		12/80	10/46	4.7/40
pNIPAAm(2) gel			0.44/3.9			

Introduction amount of guaiacol into gels

5 × 5 × 5 mm^3 (125 μL) of hydrogel was immersed in 5% EtOH in 0.02 M phosphate buffer (PB) (pH 7.2) (1.0 mL) containing 50 mM guaiacol as substrate at 26 ºC for 5 min. To estimate the amount of guaiacol incorporated into the gel, the concentration of the guaiacol in the solution was calculated from Lambert-Beer's law using the result of UV-Vis measurement. The introduction amount of guaiacol in pNIPAAm(2) gel was higher than that of pPAAm(2) gel.

$A_{274\ nm} = \varepsilon_{274\ nm} \times C_{guaiacol} \times l$

$A_{274\ nm}$ (absorbance at 274 nm)

$E_{274\ nm}$: 2.3 × 10^3 $M^{-1}cm^{-1}$

C_{produt} (concentration of the guaiacol)

l = 1 cm

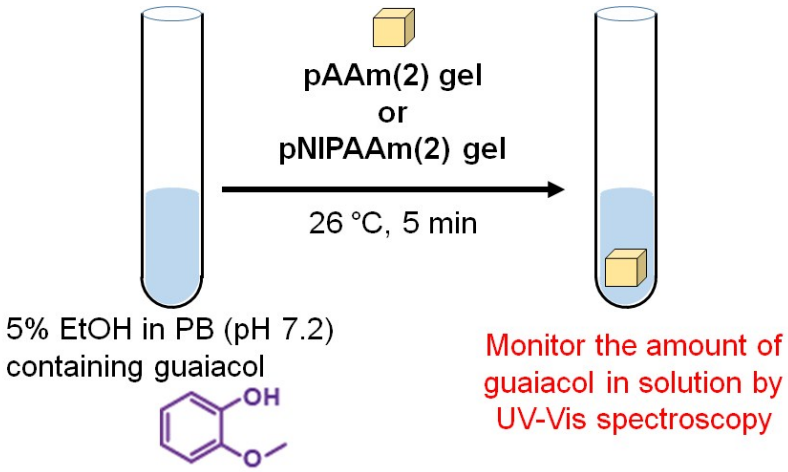


Figure S5. Experimental procedure of incorporation of guaiacol into pAAm(2) gel and pNIPAAm(2) gel.

Table S7. Introduction amount of guaiacol into the gels.

Hydrogels	Introduction amount of guaiacol into the hydrogel (μmol)
pAAm(2) gel	6.2
pNIPAAm(2) gel	12.0

Dependence of reaction rate on heptanoic acid (decoy molecule) concentration

To determine dependence of reaction rate of P450 gels on heptanoic acid concentration, we plot the reaction rate of P450 gel versus heptanoic acid concentration. There was no significant difference in the heptanoic acid concentration dependence of the reaction rate between P450-pAAm(2) gel and P450-pNIPAAm(2) gel (Fig. S4). This result indicates that the ability to incorporate heptanoic acid into P450 in the gel is almost the same.

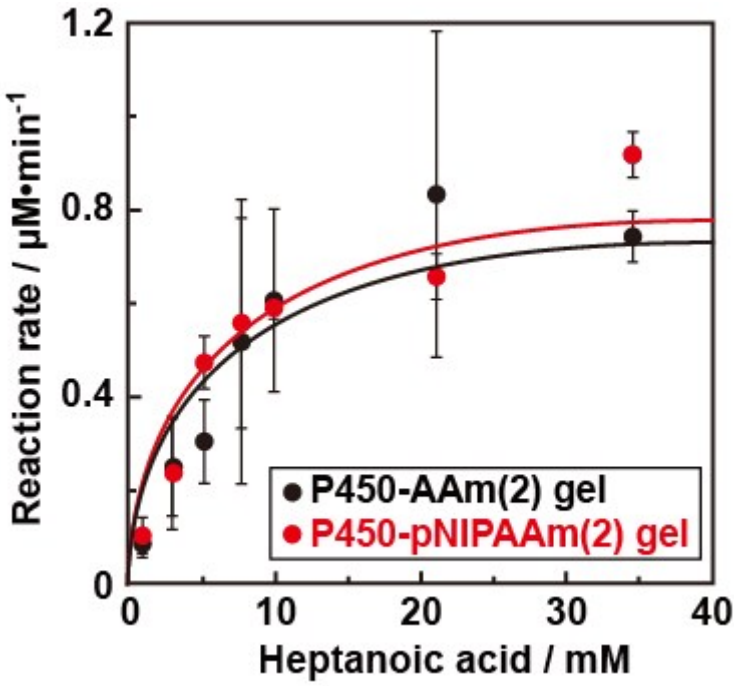


Figure S6. The concentration dependence of heptanoic acid on the oxidation reaction catalyzed by P450 in PB (pH 7.2) with 5% EtOH containing guaiacol.

Cross-linking densities of P450-pNIPAAm gel

We investigated the swelling ratio of the P450-pNIPAAm(*x*) gels with different amounts of cross-linker (MBAAm) (Table S2). The P450-pNIPAAm(*x*) gels were immersed in 5% EtOH in 0.02 M PB (pH 7.2). Swelling ratio (%) = hydrogel weight after immersion / hydrogel weight before immersion. The swelling ratio decreased as increasing MBAAm content, which shows that cross-linking density increase as increasing MBAAm content.

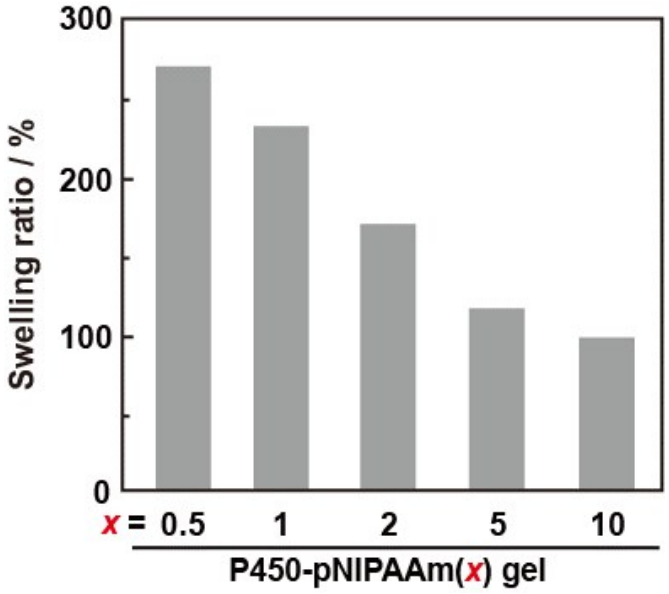


Figure S7. Swelling ratio of P450-pNIPAAm(*x*) gel.

Dependence of reaction rate of P450-pAAm(2) gel on the temperature

The reaction rate of P450-pAAm(2) gel was increased with increasing temperature from 26 to 37 ^{0}C (Fig. S8a). This is probably because of the increase in enzymatic activity with increasing temperature. At this time, the swelling ratio, namely size, of P450-pAAm(2) gel was not changed [swelling ratio (%) = (weight of gel at 37 ^{0}C / weight of gel at weight of 26 ^{0}C) × 100] (Fig. S8b).

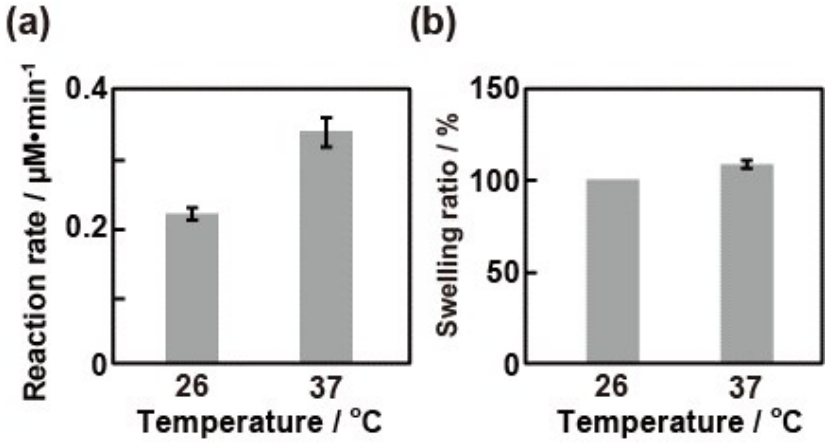


Figure S8. Temperature dependence of (a) reaction rate and (b) swelling ratio of P450-pAAm(2) gel.

Determination of LCST of P450-pNIPAAm(2) gel

In order to the determine the lower critical solution temperature (LCST) of P450-pNIPAAm(2) gel, We plot the swelling ratio versus temperature (Swelling ratio = weight of the gel after heating / weight of the gel at 24 ^{0}C) (Fig. S9). As a result, LCST was 31.5 ^{0}C.

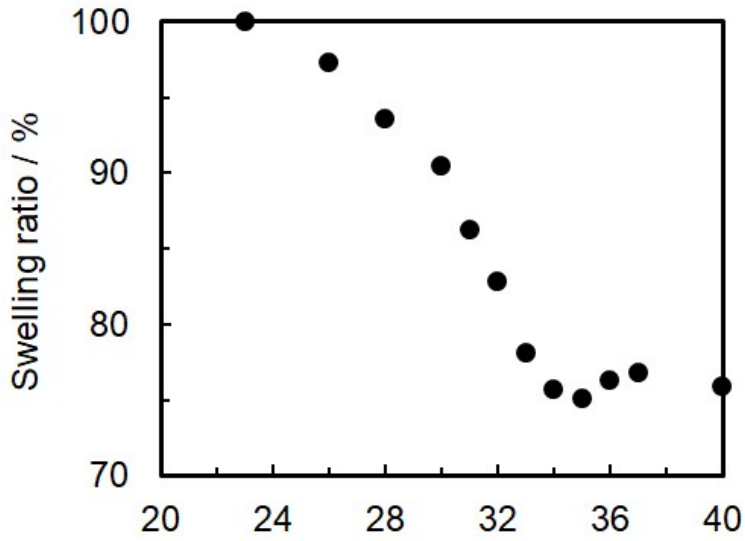
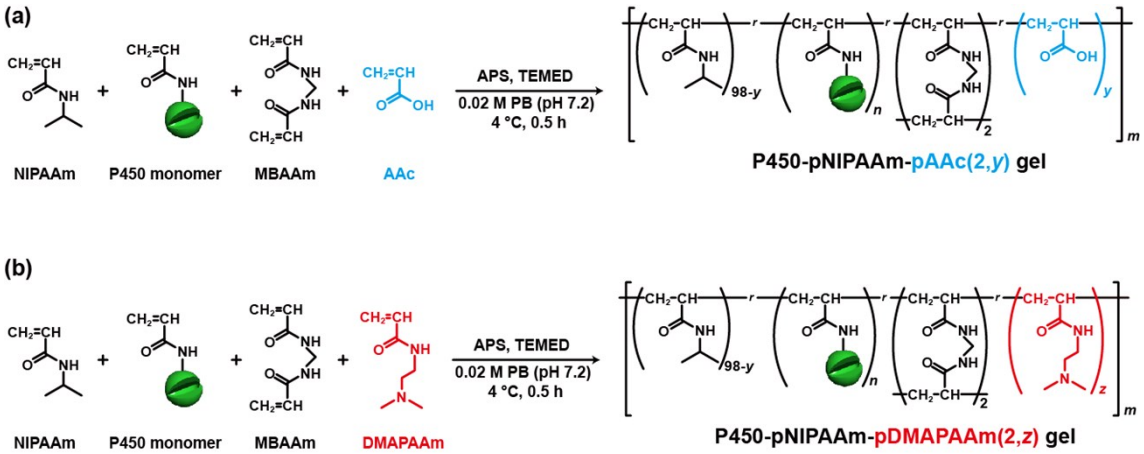


Figure S9. Plot of swelling ratio versus temperature of P450-pNIPAAm(2) gel.

Enzymatic activity of P450-pNIPAAm-pAAc gel and P450-pNIPAAm-pDMAPAAm gel

Preparation of P450-pNIPAAm-pAAc gel and P450-pNIPAAm-pDMAPAAm gel



Scheme S4. Preparation of (a) P450-pNIPAAm-pAAc(2,*y*) gel and (b) P450-pNIPAAm-pDMAPAAm(2,*z*) gel. Here, 2, *y*, *z*, and *n* represent the mol% of the MBAAm, AAc, DMAPAAm and P450 units, respectively. The mol% of P450 unit was determined to be $n = 1.3 \times 10^{-4}$.

AAc or DMAPAAm and MBAAm was dissolved in water. Upon the addition of APS and TEMED, the reaction mixture immediately became a gel (Tables S8 and S9). The gel was washed with water to remove the unreacted monomers and initiators and was subsequently soaked in an excess of 0.02 M PB (pH 7.2).

Table S8. Preparation of P450-pNIPAAm-pAAc(2,1.0) gel.

Water (mL)	P450 monomer (mg/nmol)	pNIPAAm (g/mmol)	AAc (mg/μmol)	MBAAm (mg/μmol)	APS (mg/μmol)	TEMED (mg/μmol)
2.0	0.32/6.7	0.44/3.9	2.8/40	12/80	10/46	4.7/40

Table S9 Preparation of P450-pNIPAAm-pDMAPAAm(2,1.0) gel.

Water (mL)	P450 monomer (mg/nmol)	pNIPAAm (g/mmol)	DMAPAAm (mg/μmol)	MBAAm (mg/μmol)	APS (mg/μmol)	TEMED (mg/μmol)
2.0	0.32/6.7	0.44/3.9	6.2/40	12/80	10/46	4.7/40

Reaction rate of P450-pNIPAAm gel, P450-pNIPAAm-pAAc gel, and P450-pNIPAAm-pDMAPAAm gel

The reaction rate of P450-pNIPAAm-pAAc gel was lower than that of p450-pNIPAAm(2) gel. In addition, the reaction rate of the reaction rate of P450-pNIPAAm-pAAc gel was decreased with increasing the mol content of AAc units in the gel from 0.5 to 1.0 (Fig. S10). In contrast, the reaction rate of P450-pNIPAAm-pDMAPAAm(2, 0.5) gel was higher than that of p450-pNIPAAm(2) gel. However, the reaction rate of P450-pNIPAAm-pDMAPAAm(2, 1.0) was lower than that of P450-pNIPAAm-pDMAPAAm(2, 0.5) (Fig. S10).

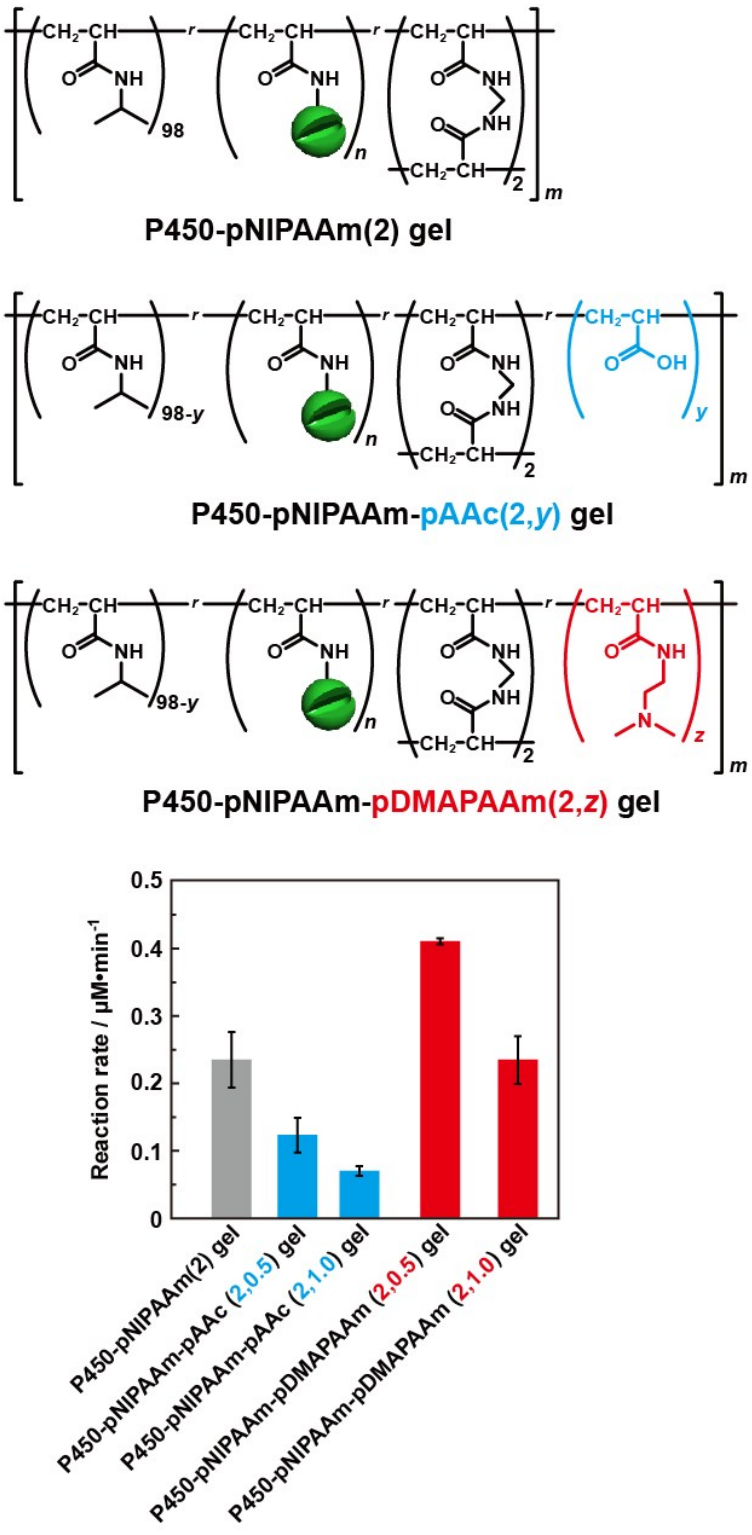


Figure S10. Chemical structures of P450-pNIPAAm(2) gel, P450-pNIPAAm-pAAc(2,*y*) gel, and P450-pNIPAAm-pDMAPAAm(2,*z*) gel and their reaction rates.

Effect of hydrophobicity on the enzymatic activity of P450-immobilized hydrogel

In order to examine the effect of hydrophobicity on the enzymatic activity of P450-immobilized hydrogel, we investigated the effect of hydrophobicity of decoy molecule on the reaction rate. The reaction rate of P450-pNIPAAm(2) gel was investigated using carboxylic acid with different alkyl chain lengths (Fig. S11). The reaction rate was highest using heptanoic acid (n = 4) as decoy molecule, which was similar to native P450.[5]

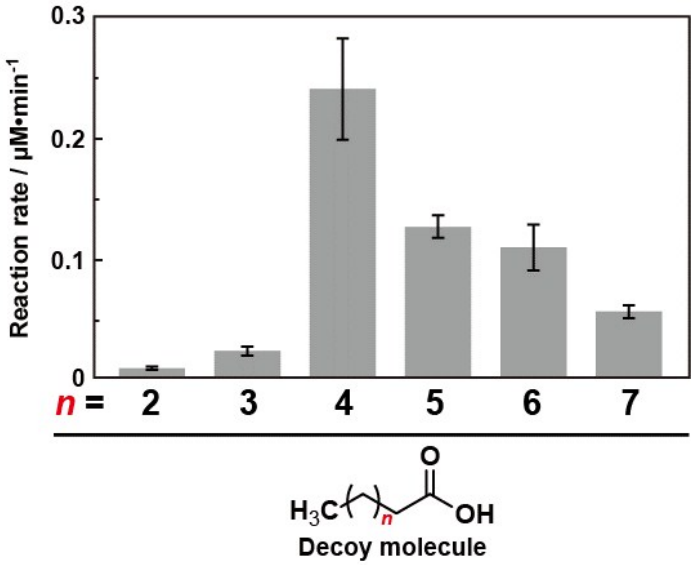


Figure S11. Reaction rate of P450-pNIPAAm(2) gel in the presence of decoy molecules with different alkyl chain lengths.

References

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