Supporting Information

In Situ Synthesis of Protein-Loaded Hydrogels *via* Biocatalytic ATRP

Aotian Zhang,[†‡] Xiancheng Meng,[†‡] Qiang Zhang[†‡*]

[†] Jiangsu Key Laboratory of Chemical Pollution Control and Resources Reuse, School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing 210094, P. R. China.

[‡] Institute of Polymer Ecomaterials, School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing 210094, P. R. China.

* Email: zhangqiang@njust.edu.cn

Experimental section

Materials

Laccase from *Trametes versicolor* (≥0.5 U/mg) was supplied by Sigma-Aldrich and purified by dialysis against water for 1 day following lyophilization. Rhodamine B 4-(3-(N-hydroxysuccinimidyloxocarbonyl)-propyl)-piperazine amide was synthesized according to previous procedures for the labeling of laccase directly.[1, 2] Tris(2-(dimethylamino)ethyl)amine (Me_6TREN) was synthesized according to literature procedure and stored in the freezer under a nitrogen atmosphere.[3] Poly(ethylene glycol) diacrylates (average M_n 2000 and 8000) and a water-soluble initiator (2-hydroxyethyl 2-bromoisobutyrate, HEBiB) were synthesized according to previous reports.[4-6] All other reagents, such as ethyl 2-bromoisobutyrate (EBiB, 98%), sodium ascorbate (NaAsc, 99%), poly(ethylene glycol) methyl ether acrylate ($PEGA_{480}$, average M_n 480), poly(ethylene glycol) diacrylate (PEGDA, average M_n 400 and 1000), *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA, 99%), 2,2′-bipyridyl (BPY, 99%), methyl acrylate (MA, 99%), dimethyl sulfoxide (DMSO), hydroquinone (99%) were obtained from Aladdin (China) and used without further purification. Membrane dialyses (1K and 50K, MWCO) were obtained from Spectrum Laboratories.

Analytical Techniques

Inductively coupled plasma mass spectrometry (Elan DRC-e, America PerkinElmer) was used to measure the concentration of the residual Cu-ions and the detection limit is less than 0.5 ppb. The enzyme activity of laccase was calculated according to the

degradation of 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in PBS buffer using SHIMADZU UV-2600 UV-Vis spectrophotometer to measure the absorbance at 420 nm. The number-average molecular weight (M_n) and molecular weight distribution (M_w/M_n) were determined by Waters 1515 size exclusion chromatography (SEC) in N,N-dimethylformamide (DMF) at 40 ℃ with a flow rate of 1.00 mL min^{-1}, which was equipped with refractive index (RI) and UV detectors, a 20 μm guard column (4.6 mm × 30 mm, 100−10K) followed by three Waters Styragel columns (HR1, HR3, and HR4) and autosampler. Narrow linear polystyrene standards in the range from 540 to 7.4×10^5 g mol^{-1} were used to calibrate the system. All samples were passed through 0.45 μm PTFE filter before analysis. Scanning electron microscope (SEM) images were acquired by FEI Quanta 250 FEG. Thermogravimetric analyses (TGA, Mettler Toledo, Switzerland) were performed under nitrogen atmosphere by heating samples from 50 ℃ to 800 ℃ at a rate of 10 ℃ / min. Rheological experiments were carried out with DMA Q800 (US TA). A frequency sweep on the hydrogel was performed from 0.01 to 10 Hz at 1% strain under 30℃.

Detection of copper concentration by ICP-MS

In order to detect the copper ion concentration accurately, the mixed standard and internal standard were used simultaneously. Among them, the mixed standard (Ambient, Quality Control Standard #26, 26 Elements, 100 mg/L in 5% HNO_3 + Tr HF) determined the standard curve, while the internal standard (China, national standard solution Rh, 1000 μg / mL, medium 10% HCl) ensured the normal operation

of the instrument.

Generally, an aqueous solution (5 mL) of laccase (30 mg) and ligand (60 μmol) in a dialysis tubing (MWCO, 50K Da) was dialyzed against water (80 mL) for 24 h before ICP-MS characterization. The samples from the solution outside of the dialysis tubing were directly determined. The background emission from pure water was compensated by the preparation of the sample and calibration solutions in the same media.

While the sample (0.1 mL) from the inside of the dialysis tubing containing pristine laccase was first mixed with 0.3 mL concentrated nitric acid (HNO_3, 70%) and heated at 100 ℃ for 3 hours and then cooled to ambient temperature before ICP-MS characterization. The background emission from nitric acid was compensated by determining a blank sample containing only nitric acid.

Synthesis of poly($PEGA_{480}$) by ARGET ATRP

In one vial equipped with magnetic stir bar and rubber stopper, EBiB (10 mg, 0.05 mmol), ligand (either PMDETA, 9.7 mg, 0.06 mmol or BPY, 8.7 mg, 0.06 mmol or Me_6TREN, 12.9 mg, 0.06 mmol), $PEGA_{480}$ (817 mg, 1.7 mmol) and water (4 mL) were charged. The solution was bubbled with nitrogen for 15 min. After that two degassed aqueous solutions containing laccase (0.1mL, 30 mg laccase) and NaAsc (0.1mL, 75 mg NaAsc, 0.38 mmol) were added into the vial separately. The reaction mixture was allowed to stir under nitrogen protection at defined temperature for 6 h. After that, the solution was transferred into a dialysis tubing (MWCD 1000 Da) to dialyze against water for 2 days. The final faint yellow polymer containing laccase was recovered *via*

lyophilization and the conversion was determined gravimetrically.

As a library of control experiments, the polymerizations were also performed without laccase but using $CuBr_2$ as the catalysts. The procedure is the same as mentioned above and only used $CuBr_2$ (0.42 mg, 1.88 μmol) to replace laccase.

Poly($PEGA_{480}$) was also synthesized from a water soluble initiator, 2-hydroxyethyl 2-bromoisobutyrate (HEBiB). The procedure is the same as mentioned above and only used HEBiB (10.6 mg, 0.05 mmol) to replace EBiB.

In-situ synthesis of laccase-loaded hydrogels by enzymatic ARGET ATRP

The procedure for the preparation of hydrogels is the same as above-mentioned. Only Me_6TREN (12.9 mg, 0.06 mmol) was used as the ligand and poly(ethylene glycol) diacrylate (1.35 mmol) with MW ranging from 400 to 8000 was used as the cross-linking reagent. The polymerization was performed under 25 ℃ for 6 h. The time to form hydrogel (denoted as gelation time) was determined using the vial tilting method.[7] No flow within 1 min upon inverting the vial was regarded as the hydrogel state. After reaction, the hydrogel was washed thoroughly with water to remove any unreacted small molecules. Final product was obtained *via* lyophilization and the conversion was determined gravimetrically.

Enzyme activity of free laccase and immobilized laccase in the hydrogels

The enzyme activity of laccase was determined using ABTS as the substrate according to previously reported procedures by UV/vis spectroscopy, typically measuring the

absorbance at 420 nm for a defined sample after defined period, and the laccase activity was deduced according to the kinetic parameters.[8, 9] The enzyme activity was determined under different temperatures including 0, 25 and 60 ℃.

The hydrogel recovered *via* lyophilization should be pre-soaked into the PBS buffer to make the hydrogel reabsorb water before test. The equation for calculation of the enzyme activity of the hydrogels is shown as follow:

$$\frac{\Delta C}{\Delta t} = \frac{\Delta E \,/\, \Delta t}{36r}\left[U\ mL^{-1}\right]$$

Where r is the mass fraction of laccase in hydrogel, which was calculated by the yield and coating rate of laccase. Δ*c* is the concentration of the sample per unit of molar concentration and Δ*E*/Δ*t* represents the activity of absorbance change (ΔE) at a specific time interval (Δ*t*). The extinction coefficient for the oxidation of ABTS at 420 nm is 36 × 10^{-3} M^{-1} cm^{-1}, and the path length of the optical cell used is 1 cm. One unit was defined as the formation of 1 mM product per minute. Here, the concentration of ABTS, free laccase and hydrogel in the ABTS assays were 264.8 mg·L^{-1}, 33.3 mg L^{-1} and 5 g L^{-1} respectively.

The determination of loading and release ratio for laccase and water uptake ratio for the hydrogels

The loading and release ratio for the laccase into or out of the hydrogels could be determined by UV/vis spectroscopy. The absorbance of rhodamine B-labeled laccase at different concentrations was first measured to make a standard curve. For the

determination of loading ratio for laccase, the hydrogel synthesized using rhodamine B-labeled laccase as the catalyst was thoroughly rinsed with water until the water no longer turned pink. Subsequently, the eluent was collected to measure the total volume and the concentration. To measure the release ratio of laccase, the efficiently rinsed hydrogel was immersed into water for 12 hours, after that the concentration of laccase in the water was measured by UV/vis photometer.

The water uptake ratio of the hydrogel was determined as follows. After immersed into water for defined periods, the swollen hydrogel was taken out and the water on the surface of the hydrogel was absorbed using a filter paper. This procedure was stopped until the weight of the hydrogel did not change. The weight of dried hydrogel obtained *via* lyophilization was defined as W_d and the weight of swollen hydrogel was defined as W_s. The water uptake ratio (R_w) of the hydrogel was calculated as follow:

$$R_w = \frac{W_s - W_d}{W_d} x100\%$$

Oxidative polymerization of hydroquinone catalyzed by laccase-loaded hydrogels

The laccase loaded hydrogel (Gel_{1000}, ~ 1.5g) was added into 50 mL acetic acid buffer (pH 5.0, 0.1 M) containing hydroquinone (2.5mmol, 275 mg, 5.5 mg/ml) and stirred at 45 ℃ for 6h. During the reaction, samples were taken every hour for characterization by UV/Vis spectroscopy.

Table S1. Enzyme activity of laccase determined by UV/Vis spectroscopy.[a]

Tem[b]	Enzyme activity ($U \cdot mg^{-1}$) in the presence of			
	None	BPY	PMDETA	Me_6TREN
0℃	0.07±0.008	0.11±0.005	0.07±0.003	0.06±0.003
25℃	0.82±0.03	0.47±0.04	0.41±0.02	0.29±0.003
60℃	0.27±0.008	0.03±0.005	0.02±0.008	0.01±0.005

[a]. A solution of laccase (7.5 $mg \cdot mL^{-1}$) was heated for 6 hours in the presence of different ligands (0.015 $mmol \cdot mL^{-1}$) under different temperatures. Sample was directly taken for the enzyme activity test using ABTS as the substrate by UV/Vis spectroscopy.

[b]. The temperature of UV cuvette during the measurement of activity was the same as the temperature during the pretreatment of laccase.

Table S2. pH of the aqueous solution of laccase after addition of different ligands.

laccase in the presence of different ligands	pH
Laccase	6.84
Laccase + BPY	6.94
Laccase + PMDETA	10.30
Laccase + Me_6TREN	10.65

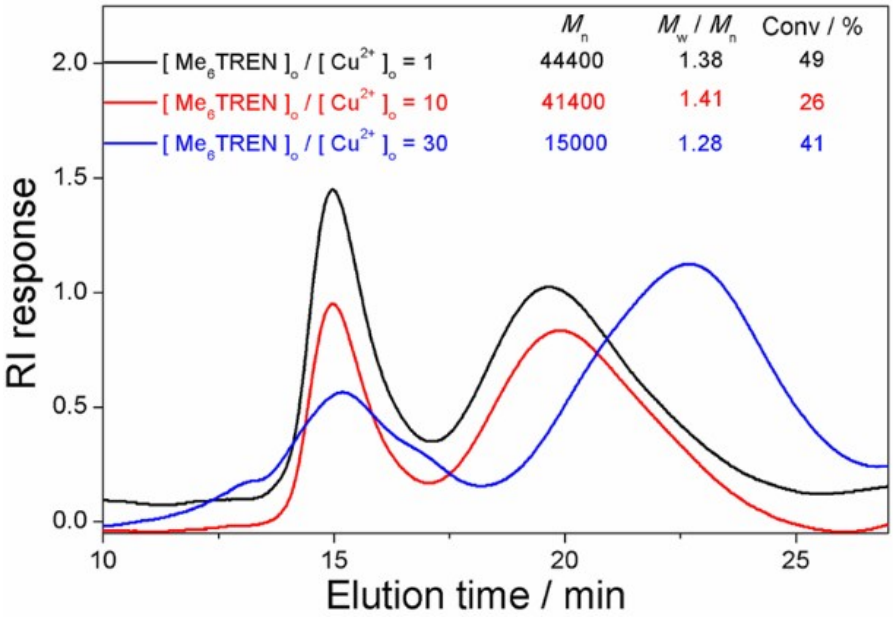

Figure S1. SEC elution traces of poly($PEGA_{480}$) prepared by ARGET ATRP at 25 ℃ in the presence of different amounts of Me_6TREN.

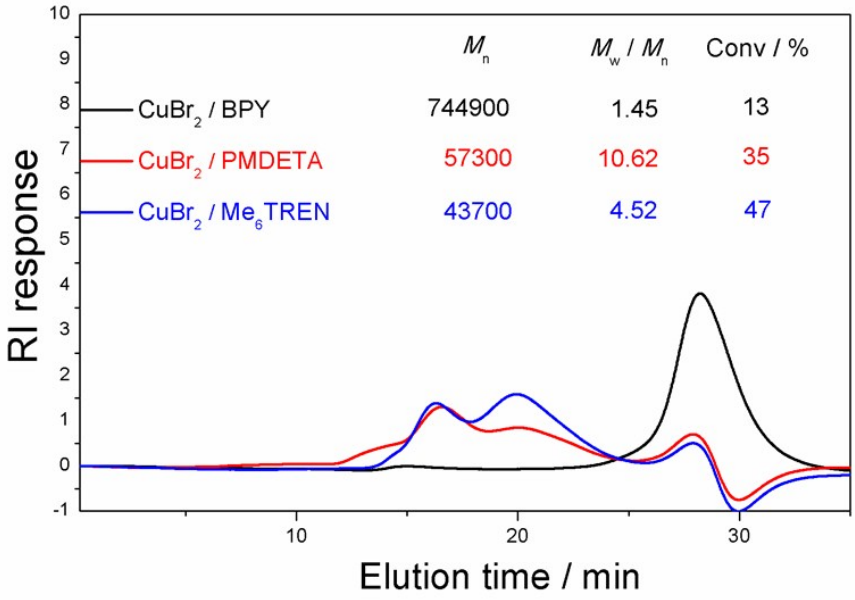

Figure S2. SEC elution traces of poly($PEGA_{480}$) prepared by ARGET ATRP using $CuBr_2$ / ligands / NaAsc as the catalyst and EBiB as the initiator.

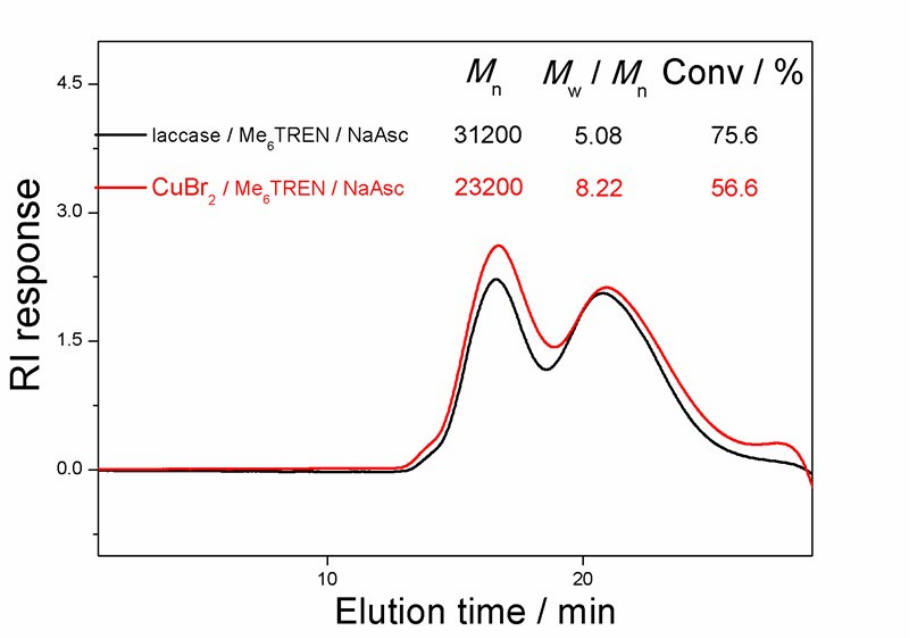

Figure S3. SEC elution traces of poly($PEGA_{480}$) prepared by ARGET ATRP using $CuBr_2$ / Me_6TREN / NaAsc or laccase / Me_6TREN / NaAsc as the catalyst and HEBiB as the initiator.

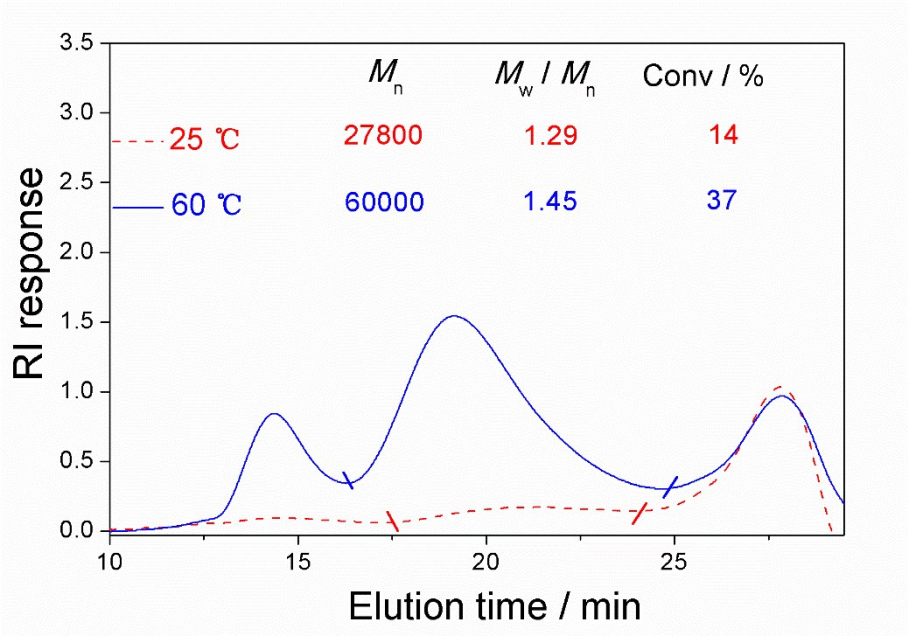

Figure S4. SEC elution traces of poly($PEGA_{480}$) prepared by ARGET ATRP in water (pH = 10.65) using laccase / NaAsc as the catalyst and EBiB as the initiator.

References

1. T. Nguyen and M. B. Francis, *Org Lett*, 2003, 5, 3245-3248.
2. D. Wang, W. Ding, K. Zhou, S. Guo, Q. Zhang and D. M. Haddleton, *Biomacromolecules*, 2018, 19, 2979-2990.
3. M. Ciampolini and N. Nardi, *Inorg. Chem.*, 1966, 5, 41-44.
4. G. Malucelli, G. Gozzelino, R. Bongiovanni and A. Priola, *Polymer*, 1996, 37, 2565-2571.
5. K. L. Shantha and N. Krishnamurti, *Journal of Applied Polymer Science*, 1989, 37, 2987-3002.
6. Rafiee and Zahra, *Amino Acids*, 48, 437-443.
7. B. Jeong, Y. H. Bae and S. W. Kim, *Macromolecules*, 1999, 32, 7064-7069.
8. A. Pich, S. Bhattacharya, H. J. Adler, T. Wage, A. Taubenberger, Z. Li, K. H. van Pee, U. Bohmer and T. Bley, *Macromol Biosci*, 2006, 6, 301-310.
9. Y. Dai, J. Niu, J. Liu, L. Yin and J. Xu, *Bioresour Technol*, 2010, 101, 8942-8947.