Supplementary Information (SI) for Materials Chemistry Frontiers.

Supplementary Information

One-Pot Synthesis of Quaternary Ammonium-Functionalized Methylene Blue-Backboned Polymer for Robust NADH Oxidation

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1. *Materials and Methods*

All reagents and solvents were commercially available and used as received without further purification unless otherwise stated. Methylene blue (MB) used in control experiments were obtained from Aladdin Biochemical Tech. Co., Ltd.. Cytochrome *c* (Cyt *c*) was bought from Shanghai Macklin Biochemical Technology Co., Ltd. Alcohol dehydrogenase (ADH) was bought from Shanghai Yuanye Bio-Technology Co., Ltd. Horseradish peroxidase (HRP, Vetec^{TM}) was purchased from Sigma Aldrich.

NMR spectra were recorded on a Bruker AMX 400 spectrophotometer with the use of residual solvent or TMS as the internal reference. ESR spectra were recorded on a Bruker EMX PLUS(PPMS) EPR spectrometer. LCMS spectra were collected using an Agilent 1260-6120 LC-MS system. The infrared spectra were collected with a Thermo Fisher Nicolet iS50 FT-IR spectrometer. UV-vis spectra were recorded on a Shimadzu UV-2600 spectrophotometer.

2. *Preparation of Materials*

Both polymer MBPS and MBP-alkyl used in this research were prepared according to the previously reported procedures.[1,2] Compound **1** was synthesized according to the procedure described in our previous report.[1] Polymer MBPN-10 was obtained based on a similar synthetic route depicted in Fig. 1b. The detailed procedures were described as follows:

*2.1 Synthesis of Compound **2***

Under N_2, to a solution of **1** (1.37 g, 3.3 mmol) in anhydrous DMF (30 mL) was carefully added NaH (0.83 g, 20.8 mmol, 60% dispersion in mineral oil). The resulting mixture was stirred at room temperature for 2 h. Then, 1,6-diboromohexane (2.44 g, 10 mmol) was added and the reaction was stirred for 24-48 h at room temperature. The reaction was monitored by TLC. After the reaction was finished, de-ionized water (30 mL) was added and the mixture was extracted with dichloromethane (20 mL×3) after stirring for 10 min. The organic phase was combined, washed with brine three times, and dried over anhydrous Na_2SO_4. The solvent was then removed under vacuum. The yellow liquid was washed with ether repeatedly to remove residual 1,6-dibromomethane. The resulting yellow liquid was finally concentrated to afford Compound **2** as a yellow tar (2.17 g, yield 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm) 8.07 (d, J = 9.4 Hz, 4H), 6.53 (d, J = 9.4 Hz, 4H), 3.36-3.34 (m, 12H), 1.63-1.33 (m, 32H). ^{13}C NMR (101 MHz, $CDCl_3$) δ(ppm) 152.51, 126.41, 115.12, 109.98, 51.33, 51.13, 33.69, 32.57, 29.52, 29.44, 27.89, 27.10, 26.99, 26.95, 26.16. MS: m/z 740.90 $[M+H]^+$, calculated for $C_{34}H_{52}Br_2N_4O_4$: 740.23

*2.2 Synthesis of Compound **4***

To a solution of **2** (1.48 g, 2 mmol) in DMF (25 mL), aqueous trimethylamine solution (30%, 15 mL) was added in one portion. The mixture was heated at 120 °C, stirred for 24-48 h, and monitored by TLC. After the reaction was complete, the mixture was cooled to room temperature. The solvent and trimethylamine were removed under vacuum. The residual was dissolved with de-ionized water (20 mL) and washed with dichloromethane for 3 times. After the removal of solvent, compound **3** was given as an orange glue (1.35 g, yield 97%) and used without further purification. MS: *m/z* 349.30 $[M]^{2+}$, calculated for $C_{40}H_{70}N_6O_4^{2+}$: 349.27.

To a solution of **3** (0.70 g, 1 mmol) in methanol (100 mL), was added Zinc powder (0.654 g, 10 mmol) and 36% HCl (5 mL). The mixture was stirred for 8 h and heated by an oil-bath of 75 °C. After the reaction was finished, the mixture was cooled to room temperature and filtrated. The filtrate was concentrated and the residual was suspended in de-ionized water, alkalified to pH ~9 with 1.0 M NaOH (aq.). The insoluble particles were then removed by filtration with a celite pad. The filtrated was concentrated to afford Compound **4** as brown tar-like material (0.339 g, yield 53%). ^{1}H NMR (400 MHz, D_2O) δ(ppm) 6.81-6.53 (m, 8H), 3.34–2.89 (m, 30H), 1.62-1.17 (m, 32H). ^{13}C NMR (101 MHz, D_2O) δ(ppm) 136.93, 130.13, 125.14, 123.55, 65.39, 58.02, 52.25, 52.06, 44.14, 26.88, 26.74, 24.28, 23.96, 23.75, 23.25, 21.04. MS: *m/z* 319.05 $[M]^{2+}$, calculated for $C_{40}H_{74}N_6^{2+}$: 319.30.

2.3 Synthesis of Polymer MBPN-10

The polymerization of compounds was conducted under the presence of ferric chloride.

To a solution of Compound **4** (0.236 g, 0.37 mmol) in 2.0 M HCl (10 mL, aq.), $Na_2S•9H_2O$ (0.74 mmol) was added and dissolved. A solution of $FeCl_3•6H_2O$ (1.11 mmol) in dilute HCl (10 mL, 1.0 M aq.) was then added. The reaction mixture was stirred at room temperature for 20 min to grantee complete polymerization. After the polymerization was finished, the mixture was dialyzed (MWCO = 3500 Da) against water for 4 days. Then the solvent was removed by lyophilization to afford polymer MBPN-10 as a dark blue solid (0.066 g, yield=28%). ^1H NMR (400 MHz, D_2O): δ(ppm) 7.24 (br, 6H), 3.68-2.75 (m, 30H), 2.03–0.82 (br, 32H).

3. *UV-Vis Study of NADH Oxidization Catalyzed by MB Derivatives*

Standard curve of MBPN-10, MBPS and MBP-alkyl were determined using UV-Vis spectra (Fig. S2a-c). For MBPN-10 and MBPS, samples at various concentration were dissolved using de-ionized water. For the water-insoluble MBP-alkyl, a 5 mg/mL DMSO stock solution was prepared and further diluted before measurement. The UV-Vis spectra were recorded at 25 °C. The absorbance of the three polymers at 680 nm was plotted against concentration. And the standard curve was thus fitted to give the standard equations: MBPN-10, $y = 0.0198x-0.00266$; MBP-alkyl, $y = 0.0287x + 0.00184$; MBPS, $y = 0.0238x-0.003$ (Fig. S2d-f). The total concentration of methylene blue motifs in solution was estimated based on the absorbance and the molecular weight of the repeating units.

Unless otherwise stated, the UV-Vis sample for the study of NADH oxidation catalyzed by MBs were prepared based on the following procedure:

To a 3 mL cuvette was added 2 mL HEPES buffer (20 mM, pH 7.4). MB derivative stock solutions (4 mg/mL aqueous solution for MBPN-10, 10 mg/mL aqueous solution for MBPS, 20 mg/mL aqueous solution for MB, and 4 mg/mL DMSO solution for MBP-alkyl) was added separately until each sample reach a final concentration of MB motif C_{MB} was 26 μM. Then NADH solution was added (20 μL, 20 mM aqueous solution) and mixed thoroughly. The mixture was then incubated for a certain period of time before UV-Vis measurement.

4. NADH Oxidization Catalyzed by MBPN-10 under Different pH and Temperature

According to the general procedure described previously, all samples with different pH were prepared and incubated at different temperature for 10 min. MES Buffer (20 mM, pH 6.0), HEPES Buffer (20 mM, pH 7.4), HEPES Buffer (20 mM, pH 8.0), and CHES Buffer (20 mM, pH 9.0) were used for samples of pH 6.0, 7.4, 8.0, and 9.0, respectively.

5. Steady-State Kinetic Analysis of NADH with MBPN-10 or MB

To an aqueous HEPES buffer (14 mL, pH 7.4, 20 mM) was added MBPN-10 (or MB) solution, until the concentration of total MB motif in solution is 26 μM. The mixture was then divided into 7 parts. Various amount of NADH solution (20 mM) was added to ensure the concentration of NADH is 10, 20, 30, 50, 100, 200 μM, respectively. The seven samples were then subjected to UV-Vis measurement immediately to record their initial UV-Vis spectra. After that, all these samples were incubated for 5 min at 37 °C. The UV-Vis spectra after incubation were then recorded.

The molar extinction coefficient of NADH is 6200 /(M•cm). The reaction velocity $V = \Delta E_{\mathrm{NADH}} / t$. After plotting C_{NADH} versus V and Michaelis–Menten fitting, the Michaelis–Menten curve was given. Whereas the Lineweaver–Burk curve was given by plotting $1/C_{\mathrm{NADH}}$ versus $1/V$ and fitted linearly. The maximum reaction V_{max} and Michaelis coefficient K_{m} was given based on the equation $1/V = (K_{\mathrm{m}}/V_{\mathrm{max}}) \times (1/C_{\mathrm{NADH}}) + 1/V_{\mathrm{max}}$. $K_{\mathrm{cat}} = V_{\mathrm{max}} / C_{\mathrm{MB}}$.

6. Catalytic Activity Stability of MBPN-10.

To a 3 mL cuvette was add 2 mL of HEPES buffer (20 mM, pH 7.4). MBPN-10 solutions (4 mg/mL aqueous solution) were added separately until each sample reached a final concentration of MB motif C_{MB} was 26 μM. Then NADH solution was added (20 μL, 20 mM aqueous solution) to give a C_{NADH}=200 μM. The mixture was incubated under air atmosphere at room temperature, and the UV-Vis spectra were recorded occasionally until complete consumption of NADH. Then an additional NADH (20 μL, 20 mM aqueous solution) was added and the change of UV-Vis spectra was also recorded. The addition was performed five times.

7. Storage and Organic Solvent Stability Study of MBPN-10.

The storage experiments were carried out by storing MBPN-10 in a 4 °C refrigerator and tested every 10 days by measuring $\Delta Abs_{338\ \mathrm{nm}}$.

For stability test in different organic solvents, a 1: 1 mixed solvent was prepared by mix various organic solvent (1 mL) with aqueous HEPES buffer (1 mL, 20 mM, pH 7.4). MBPN-10 solution and NADH solution were mixed at initial C_{MB} 26 μM and C_{NADH} 200 μM. The activity was tested by measuring $\Delta Abs_{338\ \mathrm{nm}}$ after incubation at 25 °C for 10 min.

8. ESR Experiments

To a sample (400 μL) was added HRP solution (10 μL, 0.1 mg/mL aqueous solution). DMPO (10 μL) was then added as a radical scavenger. ESR spectra were recorded with a Bruker EMX PLUS(PPMS), using a quartz sample tube. (Center field, 3518 G; Sweep width, 200 G; Sweep time, 2 min; Microwave power, 20 mW; Modulation frequency, 100 kHz).

9. Determination of H_2O_2 by HRP/TMB.

HRP (0.1 mg) was dissolved in de-ionized water (1.0 mL) to afford 0.1 mg/mL HRP solution.

TMB (4.81 mg) was dissolved in mixed solvent (1.0 mL, ethanol: DMSO = 9:1, v/v) to afford 20 mM TMB solution.

Test solution was prepared according to the UV-Vis absorption test described above (*3. UV-Vis Study of NADH Oxidization Catalyzed by MB Derivatives*).

To detect H_2O_2, HRP stock solution (10 μL) and TMB stock solution (10 μL) was added to a mixture of HEPES buffer (1.6 mL, pH 7.4, 20 mM) and test solution (400 μL). The mixture was stirred vigorously and subjected to UV-Vis test. The UV-Vis spectra were recorded to obtain the change of absorption intensity at 652 nm ($\Delta A_{652\ \mathrm{nm}}$).

For experiments under different temperature:

The concentration of generated H_2O_2 was further calculated according to the $\Delta A_{652\ \mathrm{nm}}$) and molar extinction coefficient ($3.9 \times 10^4\ M^{-1}\ cm^{-1}$) of oxidized TMB. The estimated H_2O_2 concentration was estimated to be 124.4 μM and 124.1 μM for experiment at 37 °C and 25 °C, respectively.

10. List of Figures

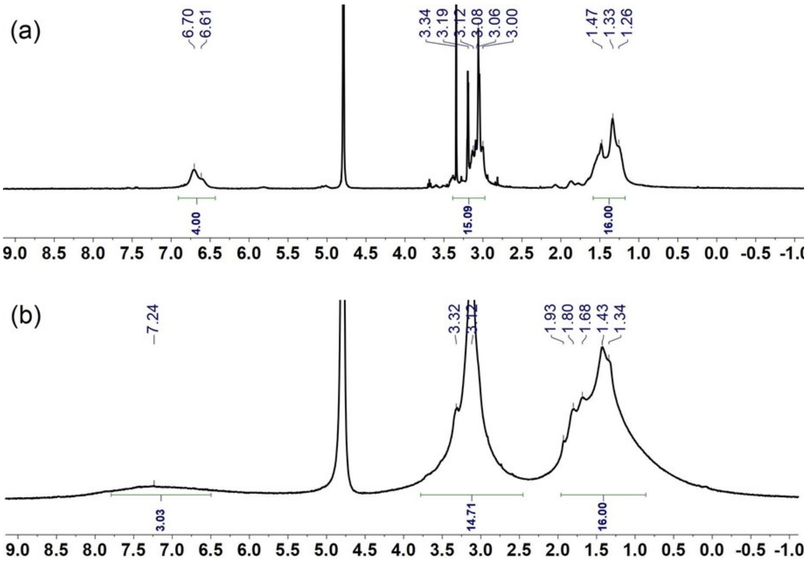

Fig S1 ^{1}H NMR spectra of (a) monomer **4** and (b) MBPN-10 (D_2O, 400 MHz, 298 K).

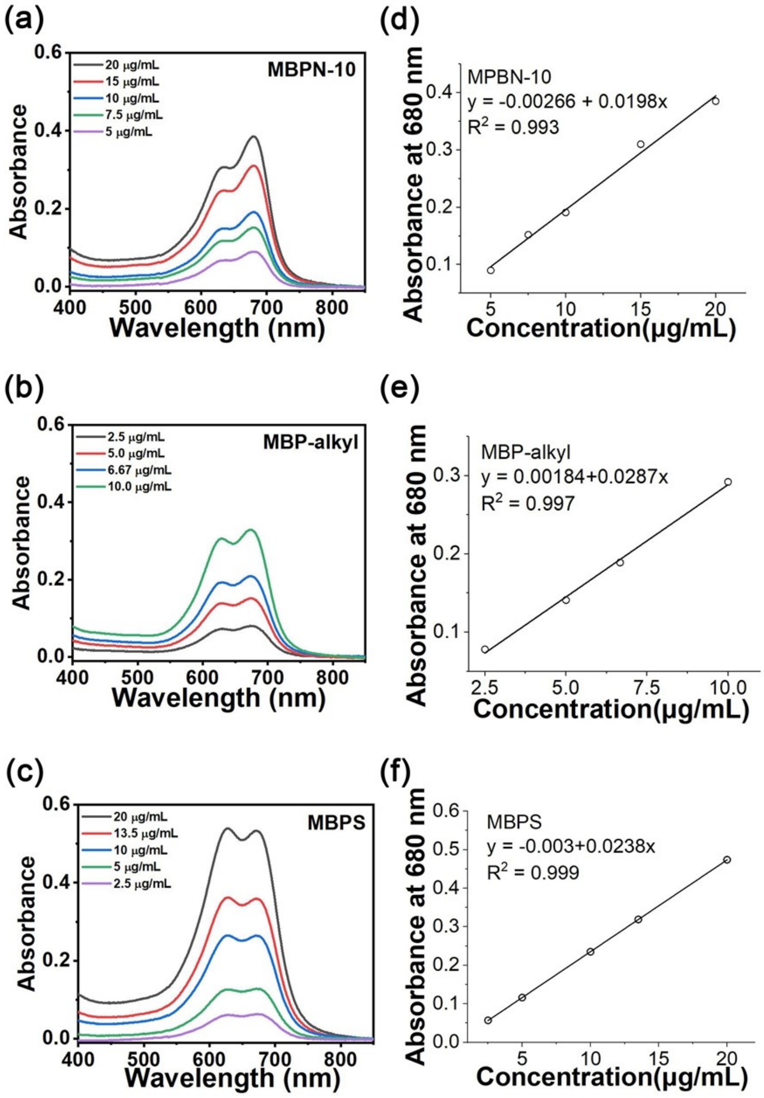

Fig. S2 (a-c) UV-Vis spectra of MBPN-10, MBP-alkyl, and MBPS at various concentrations; (d-f) Linear fitting of the absorbance of MBPN-10, MBP-alkyl and MBPS at 680 nm versus polymer concentration.

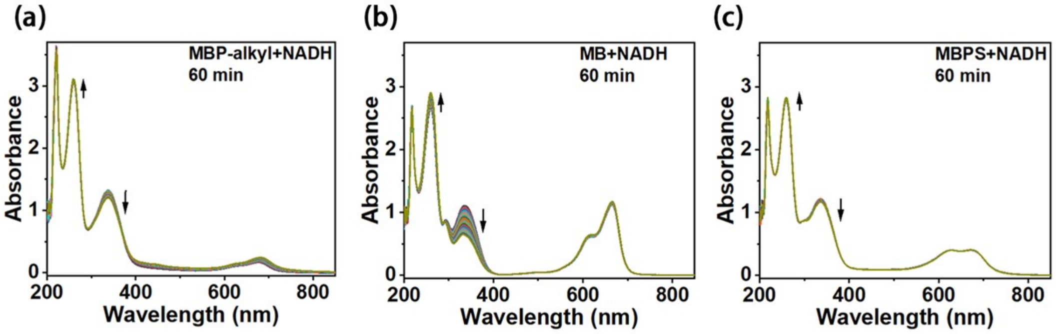

Fig. S3 UV-Vis spectral change of NADH oxidizing catalyzed by (a) MBP–alkyl, (b) MB, and (c) MBPS. Temperature, 25 °C; C_{MB}, 26 μM; C_{NADH}, 200 μM.

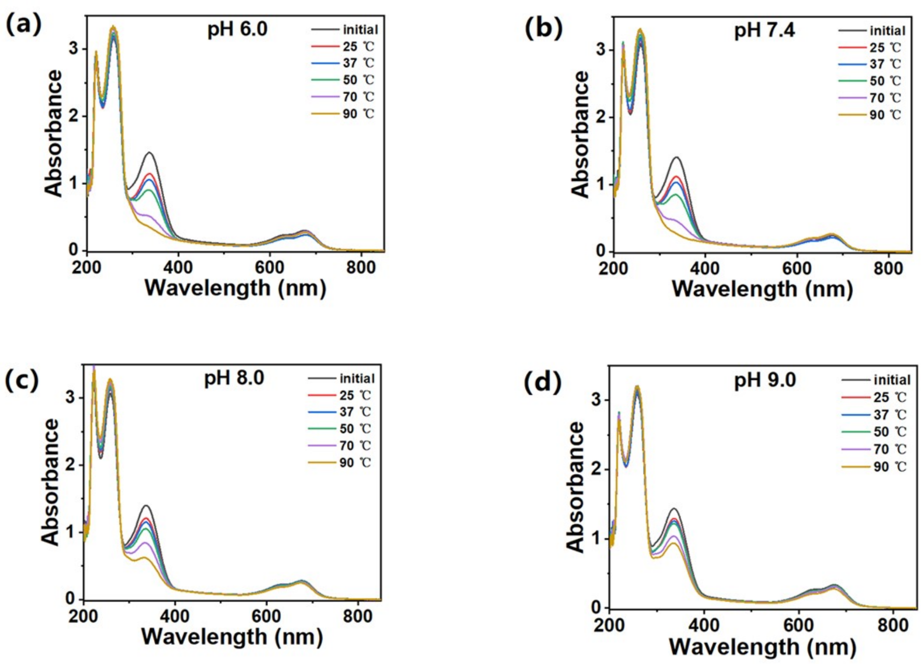

Fig. S4 (a-d) UV-Vis spectra of NADH with MBPN-10 at various pH and temperature. Incubation time, 10 min; C_{MB} = 26 μM; C_{NADH} = 200 μM.

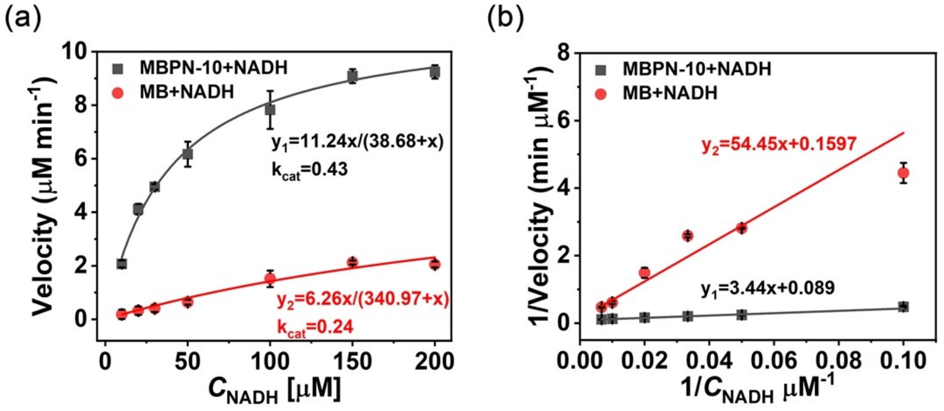

Fig. S5 (a) Michaelis–Menten curves and (b) Lineweaver–Burk curves from the steady-state kinetic analysis of NADH/MBPN-10 or NADH/MB system. Incubation time, 5 min; temperature, 37 °C; C_{MB}, 26 μM.

11. Reported Activity Data of Natural of Artificial NADH Oxidases

Table S1 Reported activity data of natural and artificial NADH oxidases at 37 °C

System	Composition	K_m, μM	K_{cat}, min^{-1}	V_{max}, μM/min	Product	Ref.
Artificial	Hemin/G–quadruplex	57.2	0.006	N. A.[a]	H_2O_2	3
Artificial	PtNP@MWCNTs	140	1.26	N. A.	H_2O	4
Artificial	Co/C	70.5	N. A.	13.9	H_2O_2	5
Artificial	PEI/ZIF–FAD	32.2	1.55	N. A.	H_2O_2	6
Artificial	PEI/ZIF–FMN	27.6	2.9	N. A.	H_2O_2	6
Artificial	Co–MoS_2	284	1.2	0.629	H_2O_2	7
Artificial	CdS/Light	2170	N. A.	20.12	H_2O	8
Artificial	Cu_xO NRs/H_2O_2	140	18.22	N. A.	H_2O	9
Artificial	Ru–MOGs/H_2O_2	340	22.31	N. A.	H_2O	10
Artificial	Cu@Cu_2O/H_2O_2	370	3.68	N. A.	H_2O	11
Artificial	G–Pd/H_2O_2	780	1.5	N. A.	H_2O	12
Artificial	MBPN-10	38.68	0.43	11.24	H_2O_2	This work
Enzymatic	Old yellow enzyme	220	42.6	N. A.	N. A.[a]	13

[a] N. A.= not available

12. Reference

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