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Development of self-cooperative nanochaperones with enhanced activity to facilitate protein refolding

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Materials

Tert-butoxycarbonyl aminoethyl poly (ethylene glycol) (Boc-NH-PEG_{114}-OH; Mw = 5000) was purchased from Yarebio Ltd. (Shanghai, China) and was dried under vacuum before use. Toluene, dichloromethane (DCM), chloroform, N, N-dimethylacrylamide (DMF) were distilled at atmospheric pressure before use. The monomer of ε-caprolactone (ε-CL, Alfa Aesear) was distilled at reduced pressure before use. Triethylamine (TEA, >99%, TCI), trifluoroacetic acid (CF_3COOH, 99.9%, Macklin), 1,10-bis(acryloyloxy)acetone (DDD, 90%, Sigma-Aldrich), 1,3-bis(4-piperidyl)propane (TDP, 98%, Sigma-Aldrich), stannous octoate ($Sn(Oct)_2$, 97%, Innochem), 3-carboxy-5-nitrophenylboronic acid (97%, J&K), O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU, 90%, ACCELA), guaiacol (98%, TCI), 5-(dihydroxyboranyl)-2-fluorobenzoic acid (98%, Macklin) and N,N-diisopropylethylamine (DIPEA, 99%, Alfa Aesar) were used directly after purchase. Lysozyme from chicken egg white was purchased from Sigma-Aldrich. Proteinase K (PK), for molecular biology, 30 U/mg was purchased from J&K. Horseradish Peroxidase (HRP) was purchased from Yien Chemical Technology Co., Ltd (Shanghai, China). Cy5-NHS ester, fluorescein isothiocyanate (FITC), and Cy3-NHS ester were purchased from Meilunbio Ltd. (Dalian, China).

Synthesis of Boc-NH-PEG-b-PCL

Boc-NH-PEG-*b*-PCL was synthesized by ring-opening polymerization in toluene using Boc-NH-PEG-OH as initiator, stannous octoate ($Sn(Oct)_2$) as the catalyst, and distilled ε-cyclohexanolactone (ε-CL) as the monomer, as follows: Boc-NH-PEG-OH (500 mg, 0.1 mmol) and ε-CL (900 mg, 7.9 mmol) were added into a dry flask, followed by the addition of 5 mL of toluene to dissolve them fully, and one drop of catalyst stannous octoate was added. After three freezing-degassing-melting cycles, the reaction was stirred at 110 ℃ for 24 hours. After termination of the reaction, the solution was precipitated with excess diethyl ether and filtrated to obtain the solid white product Boc-NH-PEG-*b*-PCL (yield, 86.1%).

Synthesis of NH_2-PEG-b-PCL

Boc-NH-PEG-*b*-PCL reacted with CF_3COOH and TEA successively to form NH_2-PEG-*b*-PCL. Briefly, BOC-NH-PEG-*b*-PCL (1 g, 0.1 mmol), CF_3COOH (5 mL), and DCM (5 mL) were added into a dry flask, and then the mixture was stirred at room temperature for four hours. The solvent and the excess CF_3COOH were removed by rotary evaporation under vacuum at 30 ℃. To remove all the CF_3COOH, 5 mL of DCM was added to re-dissolve the residue, followed by rotary evaporation again. The dissolution/evaporation cycles were repeated three times. After the last rotary evaporation, DCM (5 mL) and TEA (5 mL) were added, and the reaction was carried out at room temperature for 12 hours. Then the same rotary evaporation was carried out to remove the excess TEA and DCM, and the mixture was precipitated with excess diethyl ether and dried in a vacuum oven to obtain NH_2-PEG-*b*-PCL (yield, 94.0%).

Synthesis of PBA-PEG-b-PCL

The synthesis of PBA-PEG-*b*-PCL was obtained by the reaction of NH_2-PEG-*b*-PCL with phenylboronic acid. Briefly, phenylboronic acid (15 mg, 0.08 mmol), DMF (5 mL), HATU (46 mg, 0.12 mmol), and DIPEA (20 μL) were added into the reaction flask, and the mixture was stirred at room temperature for 1 h to activate the carboxyl group. Subsequently, vacuum-dried NH_2-PEG-*b*-PCL (50 mg, 0.005 mmol) was added, and the reaction was carried out overnight at 40 ℃. After the reaction, the mixture was dialyzed (molecular cut off: 5 kD) to remove excess PBA and PBA-PEG-*b*-PCL eventually obtained by lyophilization (yield, 95.6%).

Synthesis of PCL-b-PDAE

The synthesis of PCL-*b*-PDAE was accomplished by ring-opening polymerization and Michael addition polymerization reactions. Firstly, PCL with acrylate at the end group (PCL-A) was synthesized by using 2-hydroxyethyl acrylate as the initiator, ε-CL as the monomer and $Sn(Oct)_2$ as the catalyst, and the synthesis method was the same as that of Boc-NH-PEG-*b*-PCL. Then, PCL-A (638 mg, 0.1 mmol), TDP (315 mg, 1.5 mmol), DDD (423 mg, 1.5 mmol) and chloroform (10 mL) were added into a round-bottomed

flask under a nitrogen atmosphere, and the mixture was stirred at 55 ℃ for 72 h. After the reaction, the mixture was precipitated with excess diethyl ether, filtered and dried to obtain PCL-*b*-PDAE (yield, 78.4%).

Synthesis of polymeric micelles

The nanochaperone (nChap) were obtained by nano-coprecipitation. Briefly, 5 mg/mL of Boc-NH-PEG-*b*-PCL, PBA-PEG-*b*-PCL, and PCL-*b*-PDAE were dissolved in anhydrous DMF before the mixed solutions in various ratios were created in accordance with Table S1. The mixture was then slowly dropped into acidic water (pH 5) while being treated with ultrasonic waves for an additional 40 minutes. To eliminate DMF, the solution was dialyzed in PB (pH 7.4) for 2 days (Mw = 5000), and then nChap were obtained.

Characterization

Proton nuclear magnetic resonance (^{1}H NMR) analysis was performed using a Bruker Avance III 400 MHz NMR spectrometer, using $CDCl_3$ and DMSO-d6 as solvents to dissolve the polymer. The polymer concentration used was 0.25 mg/mL. The morphology of the nChap was characterized by transmission electron microscopy (TalosF200C, FEI, USA), and the samples were negatively stained with 2% uranyl acetate. Circular dichroism (CD) measurements were performed on a Bio-LogicMOS-500 (France). The samples were measured in a quartz cell (0.1 mm) with a wavelength set between 190 nm and 250 nm. Secondary structure analysis was performed using CDpro software. Zeta potential measurements were performed on Brookhaven Zeta-PALS (Brookhaven Instrument, USA) after samples were stabilized in PB buffer solutions (pH 5, 6.5, 7.4). QCM-D measurements were going on a Q-Sence E4 system (Q-Sence, Västra Frölunda, Sweden).

DLS and SLS Measurements of Nanochaperone

DLS and SLS measurements were performed on a laser scattering spectrometer (BI-200SM) equipped with a digital correlator (BI-10000AT) and a 532 nm laser source. Strict dedusting was carried out before sample measurement. The nChap solution was

filtered through a 0.45 μm Millipore filter into clean scintillation vials while the HRP solution was filtered through a 0.2 μm Millipore filter into clean scintillation vials. Then, the size of individual micelles was determined. Afterward, 1950 μL of filtered nChap solution and 50 μL of HRP solution were combined, and SLS data was recorded at various intervals.

Determination of adsorption capacity

The amount of HRP adsorbed by nChap was measured using a fluorescence spectrometer (F-7000, Hitachi, Japan). Firstly, 150 μL FITC (5 mg/mL) was added dropwise to 3 mL HRP solution (5 mg/mL) to prepare FITC-labeled HRP. The mixture was dialyzed for 2 days (Mw = 5000) after being agitated at 4 ℃ for 10 hours to obtain FITC-HRP. After that, 40 μL FITC-HRP solution (5 mg/mL) was added to 960 μL nChap solutions (0.5 mg/mL), and the mixture was incubated at 25 ℃ for 6 hours. The solution was then centrifuged with an ultrafiltration tube for 20 minutes at 8000 rpm (Millipore, 100 kD). The unattached proteins were isolated, and a fluorescence spectrometer was used to calculate the adsorption capacity.

Förster resonance energy transfer (FRET)

The binding of the PDAE hydrophobic macrodomain to HRP was studied by the Förster resonance energy transfer (FRET) effect from Cy3 to Cy5. Cy5-labeled nChap and Cy3-labeled HRP (Using the same fluorescent labeling method as FITC) were incubated for 12 hours and then excited at 515 nm. The fluorescence emission spectrum in the range of 550-750 nm was recorded by fluorescence spectrometer.

Study on the interaction between phenylboric acid and HRP

The nChap were centrifuged to create a 1 mg/mL solution. Both the ARS solution and the HRP solution, with a 5 mg/ml concentration, were made simultaneously. Subsequently, using 469 nm as the excitation wavelength, 1 mL of nChap solution was added to a quartz fluorescence cell to measure the fluorescence emission intensity in the 500–800 nm range. Thereafter, 1 μL of ARS solution was added to the fluorescence cell each time, and it was incubated at room temperature for 10 minutes until the

intensity of the fluorescence at the wavelength of maximum emission (586 nm) could no longer be raised. Eventually, 10 μL of HRP solution was added to the nChap and ARS mixed solution, and the fluorescence intensity change over time was observed.

Refolding of Thermally Denatured HRP and Lysozyme

A 5 mg/mL HRP or lysozyme solution was initially prepared in 10 mM sodium phosphate buffer solution (pH 7.4). Then, 10 μL of the above solution was added to 990 μL of nChap solution respectively, and the mixed solutions were incubated at 25 ℃ for 6 hours to completely integrate the proteins with the hydrophobic microdomain of nChap. To denature HRP or lysozyme, the combined solutions were then heated at a higher temperature for a predetermined amount of time. Finally, the pH of the mixed solutions was adjusted to 5, then the mixture was placed at 4 ℃ overnight to renature the enzymes. The following was the procedure used to test the enzyme activity of refolded proteins.

Assay for the enzyme activity of various proteins

The HRP activity was determined by ultraviolet spectrophotometer. Briefly, guaiacol was dissolved at 5 mmol in 10 mM PB (pH 5) to act as the substrate solution. The guaiacol solution (1.8 mL), hydrogen peroxide (4 μL) and HRP solution (60 μL, 0.05 mg mL^{-1}) were added successively into 1 cm cuvette. Rapid mixing was followed by the measurement of the oxidation product's absorbance at 436 nm for a period of two minutes with a one-second interval using a spectrophotometer. The typical formula below is used to determine the remaining enzyme activity:

$$Recovered\ enzayme\ activity = \frac{A}{A_0}$$

In the formula, A is the slope for the first 1 min of sample after different temperature treatments, and A_0 is the slope for the first 1 min of native HRP without heating.

The enzyme-linked immunosorbent assay (ELISA) technique was used to determine IgG's ability to bind to the antigen. BSA in PBS, 200 ng/mL, 100 μL/well, was used to cover 96-well plates overnight. Following the removal of the BSA solution, the

remaining sites were blocked for two hours at 37 ℃ using a 5% skim milk solution incubation (200 μL/well). The plate should then be cleaned five times with 0.05% PBST solution after the skim milk solution has been removed. The 96-well plate received 50 μL of IgG solution in each well, and it was then incubated at 37 ℃ for two hours. Afterward, the IgG solution was eliminated, and the plate was carefully cleaned. The 96-well plate was filled with 50 μL of HRP-conjugated rabbit anti-goat IgG antibody, which had been diluted 1:2000 with PBS solution. The plate was then incubated for 45 min at 37 ℃. All wells were rinsed five times with the PBST solution after the solution was removed. After that, 100 μL of TMB was added to each well, and the reaction was allowed to proceed at room temperature for 25 minutes while being shielded from light. The reaction was terminated by the addition of 2 N H_2SO_4 (50 μL/well), and the absorbance at 450 nm was measured using a microplate reader.

Secondary structure changes of HRP

Circular dichroism (CD) was used to determine the changes in secondary structure of HRP in the presence or absence of nanochaperones after incubation at elevated temperatures. Mixed solutions of HRP and nanochaperones as well as solutions containing only HRP were first prepared and incubated at pH 7.4, 85 ℃ for 1 h, followed by overnight incubation at pH 5.0, 4 ℃ and separation of HRP and nanochaperones using ultrafiltration tubes (100 kDa). The underneath solution was collected and added to a quartz cuvette, and the circular dichroism was then used to determine the spectra in the wavelength range of 190-250 nm at a scanning rate of 100 nm/min, with a slit width of 2 nm and an optical range of 1 mm. Additionally, the measured CD spectra were simultaneously quantitatively analyzed using the CDPro software to determine the proportions of the α-helix, β-sheet, β-turn and random coil. The results obtained were averaged over three measurements and calculated.

Long-term storage of HRP

First, HRP was dispersed in a 10 mM sodium phosphate buffer solution (pH 7.4) at a concentration of 2 mg/mL. Then, 25 μL of the above solution was added to 975 μL of

different nChap solutions. The obtained mixed solutions were subsequently incubated at 40 ℃ for certain times, and the refolded proteins' activity was measured at different time points.

Kinetic Constant

The Michaelis-Menten reaction kinetics were determined based on the initial reaction rate method. In the Michaelis-Menten equation ($V_0 = V_{max}$ [S]/([S] + K_m)), several apparent kinetic parameters were defined. Hydrogen peroxide and guaiacol were selected as reaction substrates, and the substrate concentration was expressed as [S]. The Michaelis-Menten constant (K_m) indicated the affinity between the enzyme and the substrate. Finally, V_0 and V_{max} were the initial reaction velocity and the maximum velocity, respectively. The increase in the oxidation products of guaiacol determined the reaction rate. Determination of the initial reaction rate at different substrate concentrations enabled the calculation of K_m and V_{max} values.

Phenolic compound Removal Determination

Colorimetric determination of phenolic compound concentration was performed using the reaction between the sample and 4-amino antipyrine. The concentration of immobilized and free HRP was 0.5 mg/mL, added to 20 mL of phenolic compound solution (10 mg/mL), and 4 μL of 30% H_2O_2 was added to start the reaction. After an indicated time, 1 mL sample was removed, and 50 μL of 4-amino antipyrine (2.0 mM) and potassium ferricyanide reagent (6.0 mM) were added to analyze phenolic content. Spectrophotometric measurements were carried out at 510 nm after 15 min incubation at room temperature.

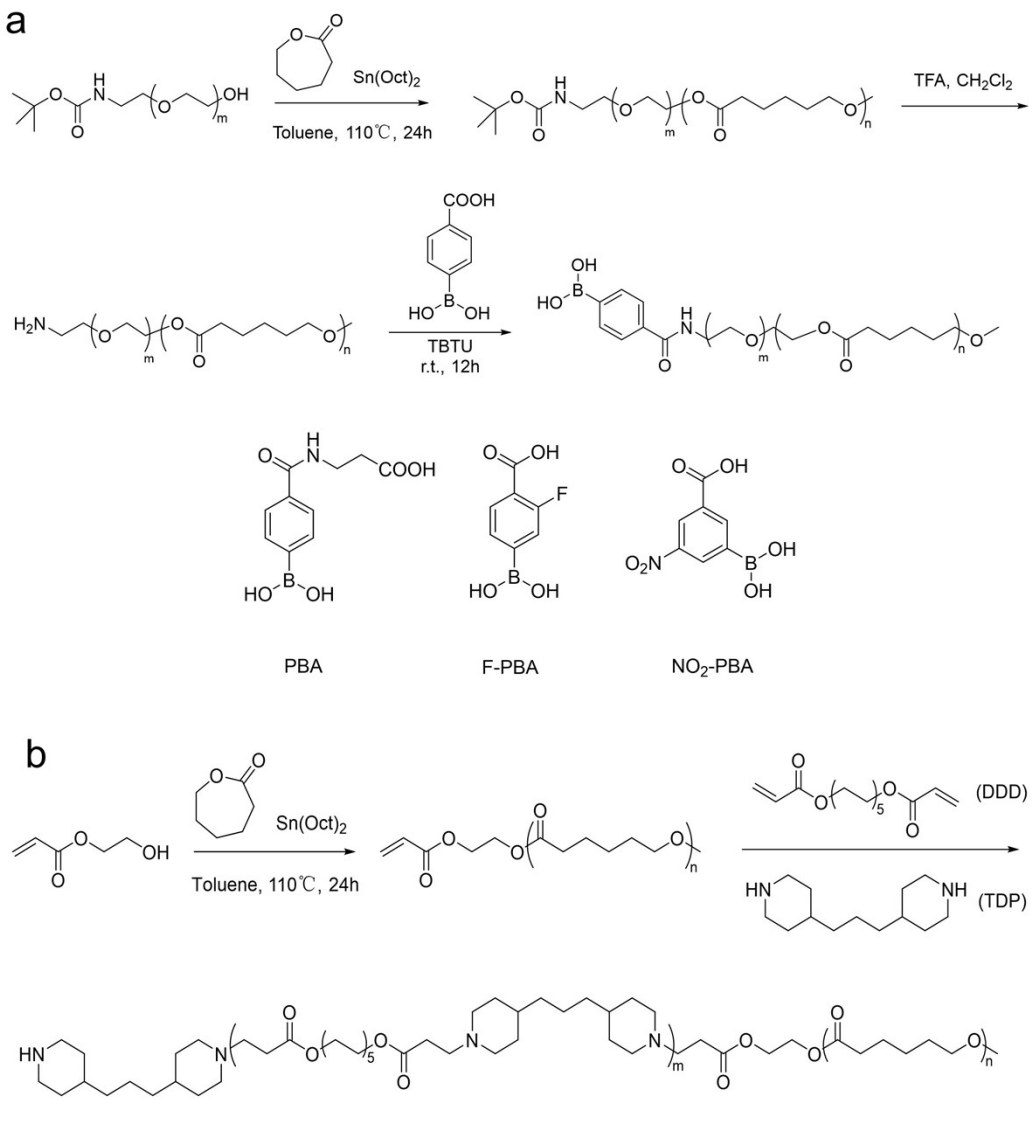

Figure S1. Synthesis of PBA-PEG-*b*-PCL (a) and PCL-*b*-PDAE (b).

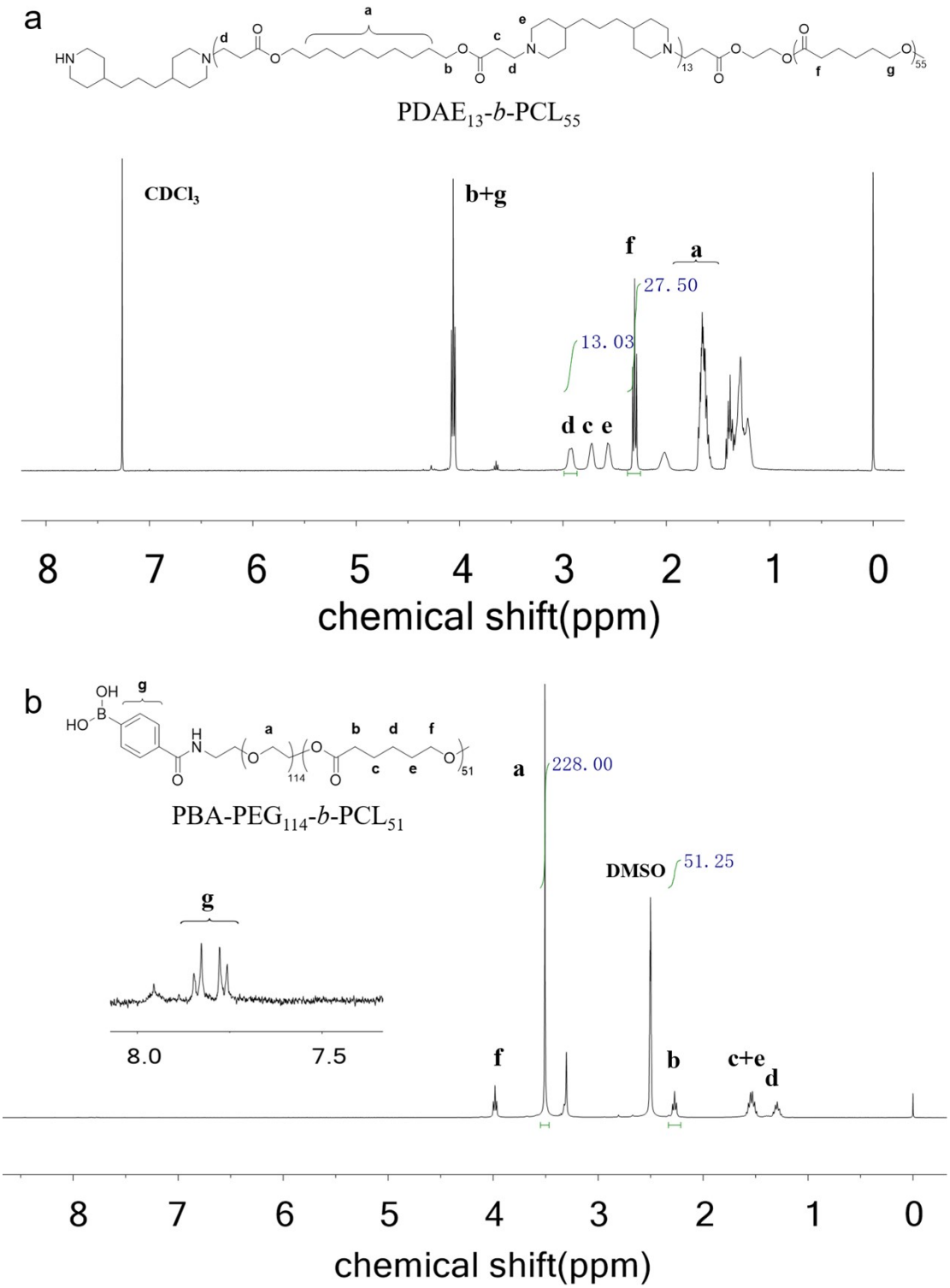

Figure S2. ^{1}H NMR spectrum of PCL-*b*-PDAE (a) and PBA-PEG-*b*-PCL (b).

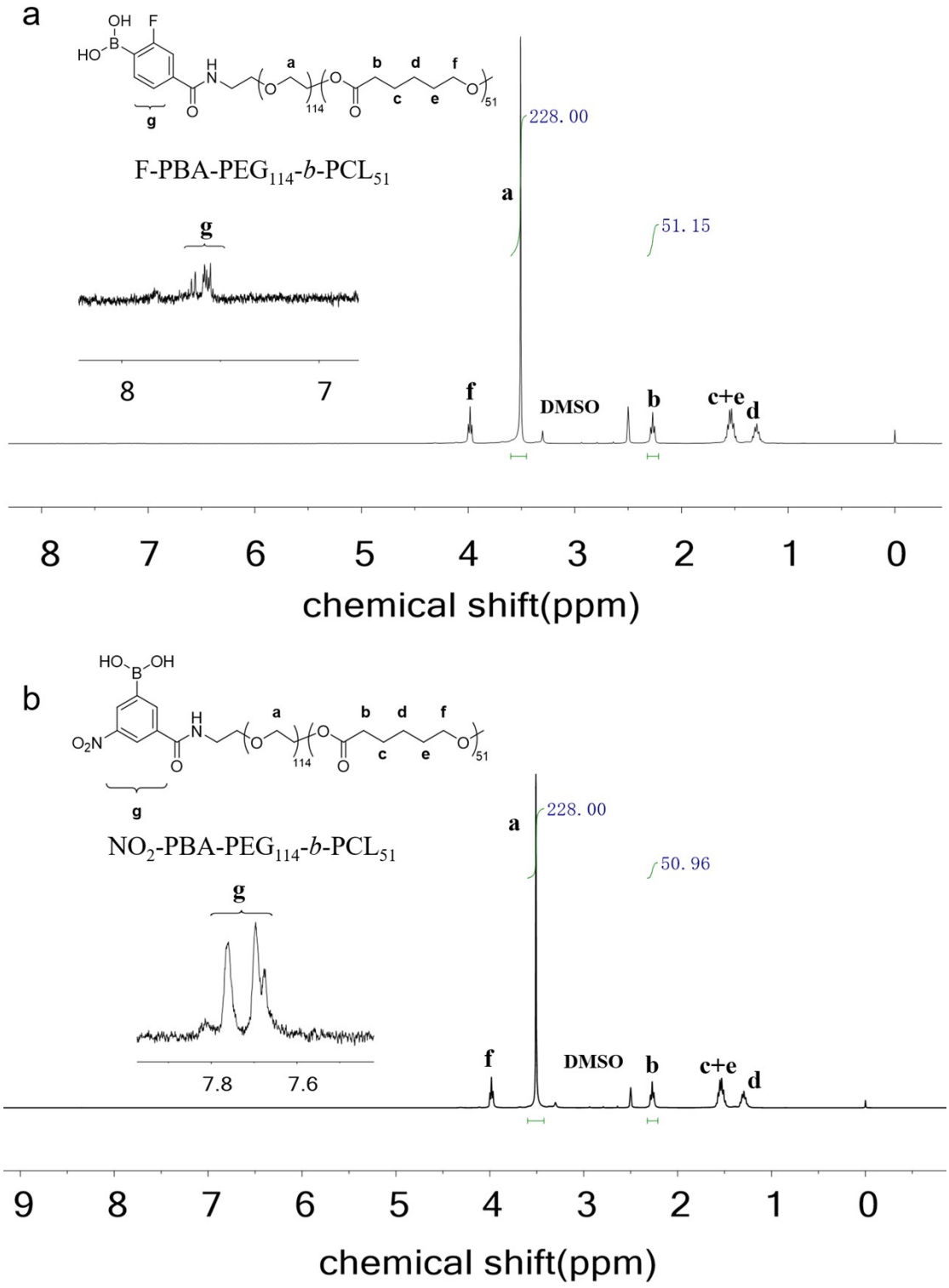

Figure S3. ^{1}H NMR spectrum of F-PBA-PEG-*b*-PCL (a) and NO_2-PBA-PEG-*b*-PCL (b).

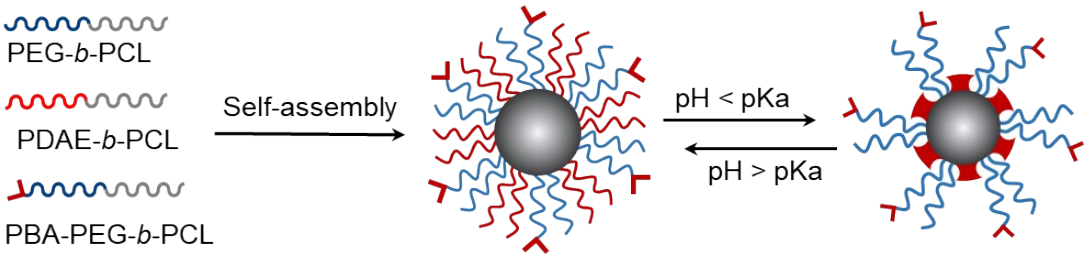

Figure S4. Schematic illustration of the preparation of MSPMs.

Table S1. Polymer composition ratios of different nanochaperone.

	PEG-*b*-PCL（w %）	PDAE-*b*-PCL（w %）	F-PBA-PEG-*b*-PCL（w %）	PBA-PEG-*b*-PCL（w %）	NO_2-PBA-PEG-*b*-PCL（w %）	Diameter (nm)	PDI	Zeta potential (mV) (pH=5)	Zeta potential (mV) (pH=7.4)	Recovery yield (%)
MSPM-1	40	50	10	0	0	81	0.16	3.64	-3.75	80 ± 2
MSPM-2	40	50	0	10	0	72	0.13	3.08	-3.83	66 ± 4
MSPM-3	40	50	0	0	10	73	0.10	3.66	-3.18	66 ± 3
MSPM-4	50	50	0	0	0	92	0.19	3.36	-3.64	45 ± 1
MSPM-5	47	50	3	0	0	76	0.18	2.84	-3.17	53 ± 3
MSPM-6	45	50	5	0	0	83	0.13	3.14	-3.27	57 ± 4
MSPM-7	30	50	20	0	0	78	0.09	4.21	-2.57	59 ± 1
MSPM-8	60	30	10	0	0	110	0.19	2.86	-3.20	64 ± 1
MSPM-9	50	40	10	0	0	78	0.14	2.83	-2.50	71 ± 3
MSPM-10	30	60	10	0	0	82	0.09	3.99	-4.24	73 ± 1
MSPM-11	20	70	10	0	0	117	0.19	4.39	-3.26	59 ± 2

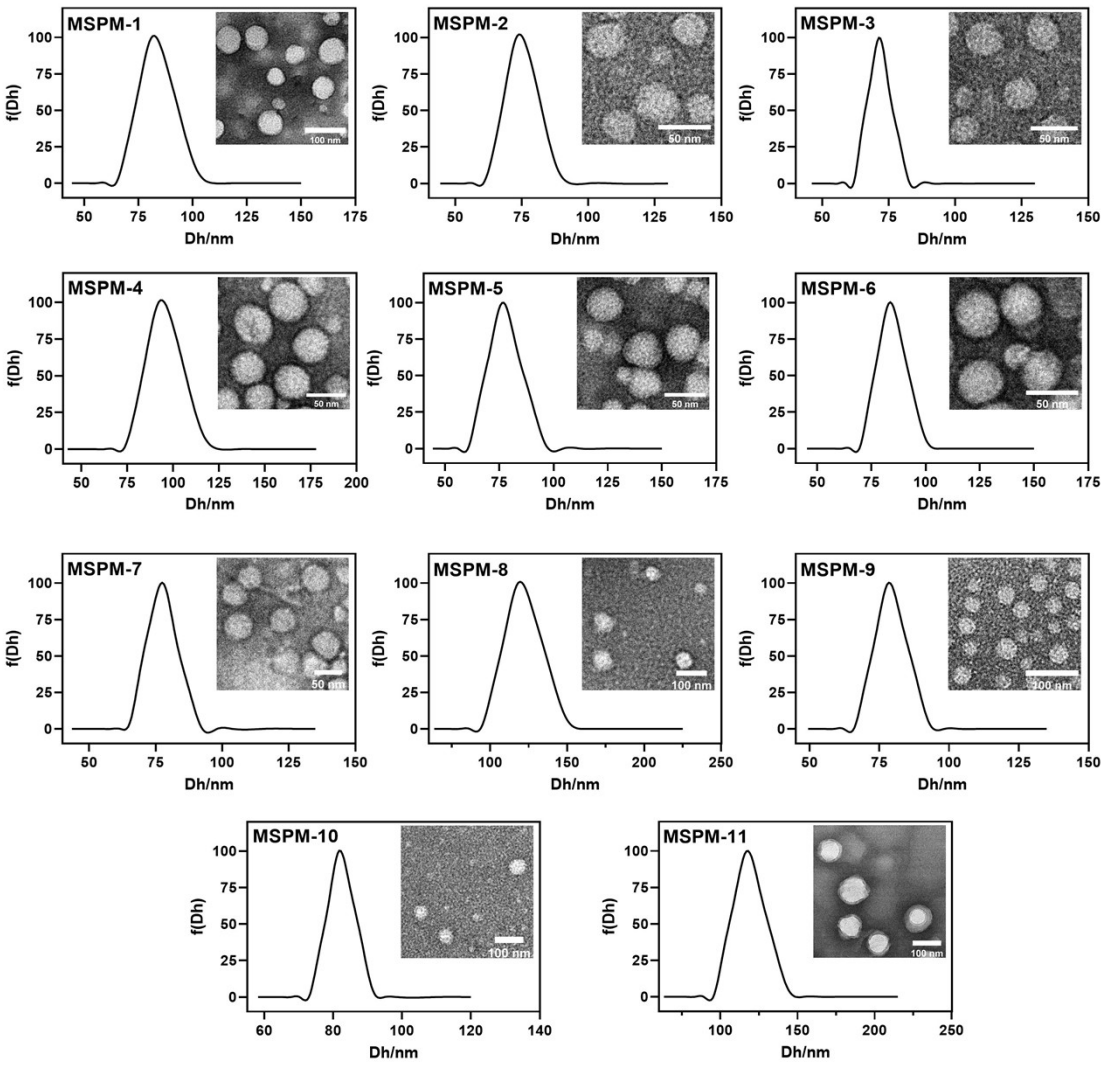

Figure S5. The morphology and size characterizations of the synthesized nanoparticles.

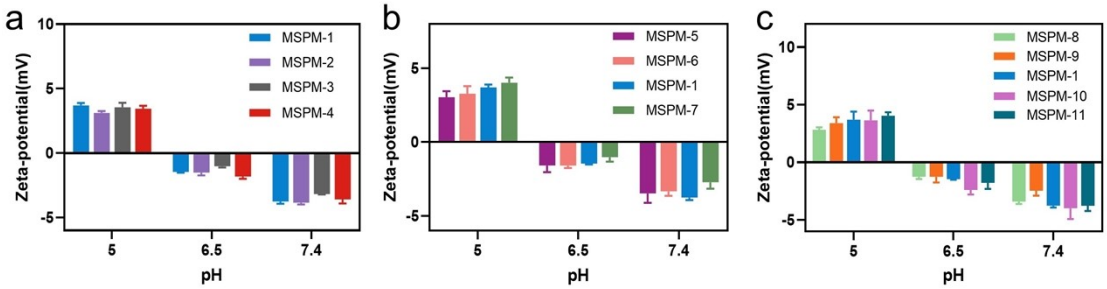

Figure S6. The zeta potential of the synthesized nChap measured at pH 5, 6.5, 7.4.

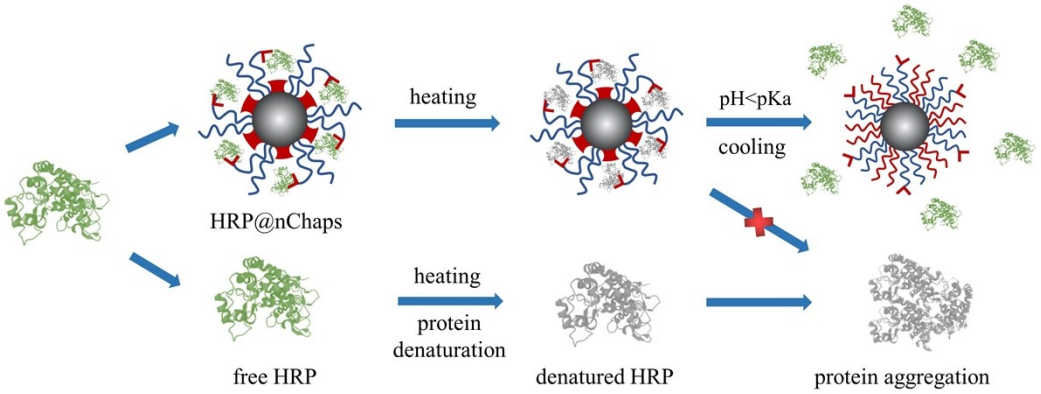

Figure S7. Schematic representation of nChap protecting proteins from aggregation inactivation.

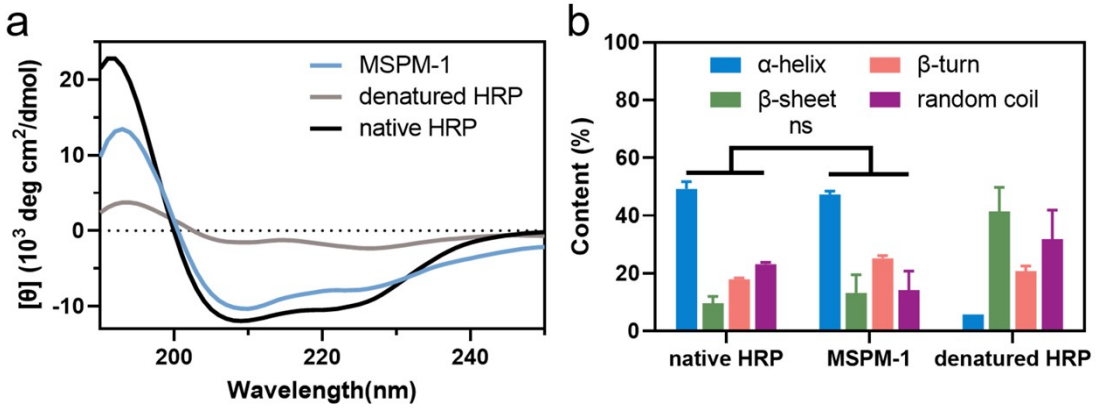

Figure S8. (a) Circular dichroism spectroscopy analysis of the native HRP and the recovered HRP in the presence or absence (control) of MSPM-1. (b) The content of various types of secondary structures of native HRP and the recovered HRP. Statistical significance was analyzed by one-way ANOVA with Dunnett's multiple comparisons test.

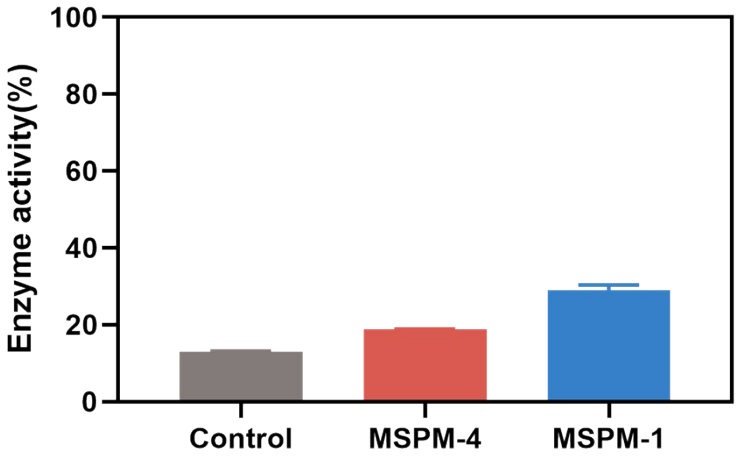

Figure S9. The recovered enzyme activity of thermally denatured HRP (50 μg mL^{-1}) in the absence (control) or presence of MSPMs (0.5 mg mL^{-1}) after being heated at pH 5.0, 85 °C for 1 h.

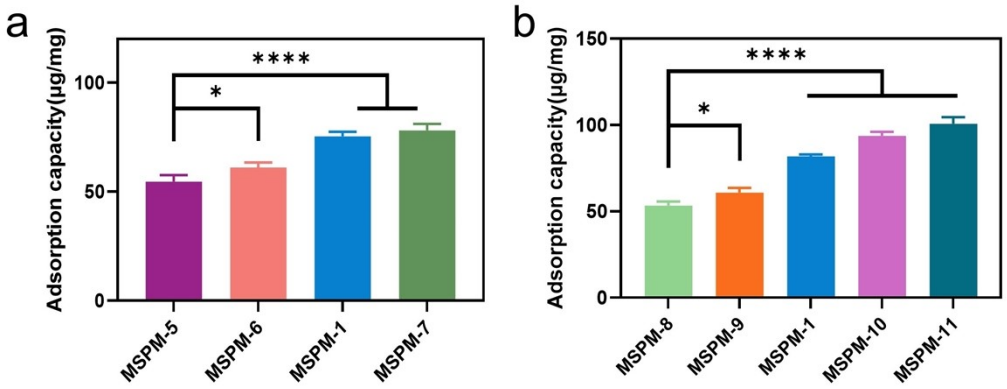

Figure S10. The measurement of the adsorbed HRP by fluorescence spectroscopy, after incubation of the nChap (0.5 mg mL^{-1}) and HRP (0.2 mg mL^{-1}) at 25 °C for 6 h. All data are represented as the mean ± SD (n = 3), statistical significance is determined by one-way ANOVA, *$P < 0.05$, and ****$P < 0.0001$.

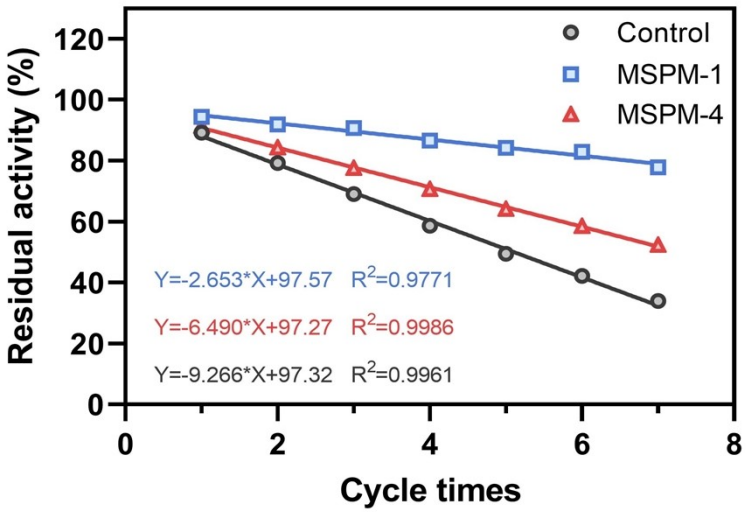

Figure S11. Linear regression analysis of the residual enzyme activity of HRP (0.05mg mL^{-1}) in the absence (control) or presence of self-cooperative nChap and non-specific nChap (0.5mg mL^{-1}) after cyclic heating at 60 ℃ for 1 h and 25 ℃ for 1 h.

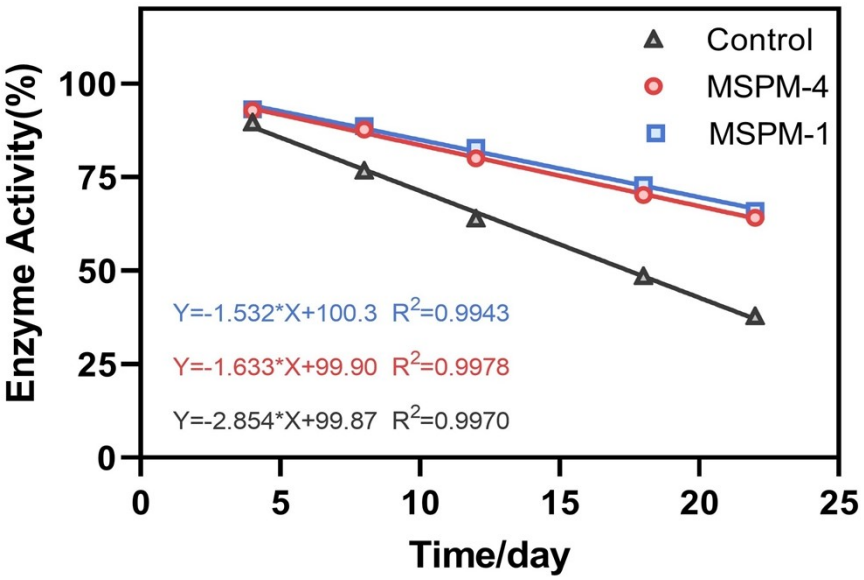

Figure S12. Linear regression analysis of the residual enzyme activity of HRP (50 μg mL^{-1}) in the absence (control) or presence of self-cooperative nChap and non-specific nChap (0.5 mg mL^{-1}) after being heated at 40 ℃ for different times.

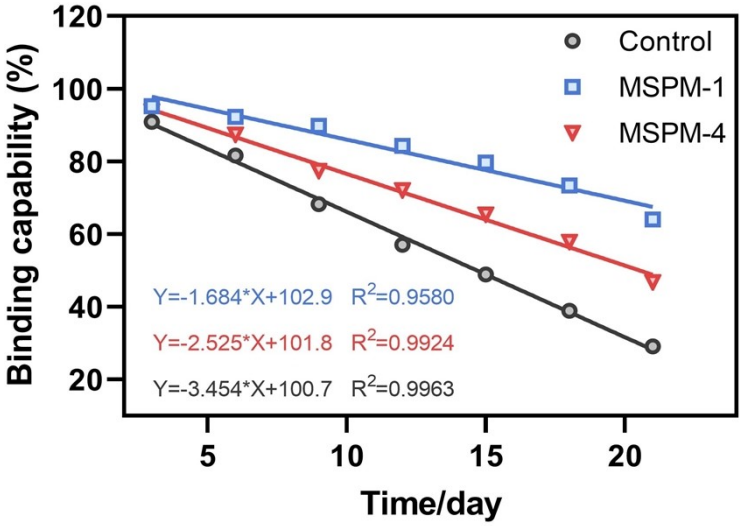

Figure S13. Linear regression analysis of the residual binding capability of G-IgG (50 μg mL^{-1}) in the absence (control) or presence of self-cooperative nChap and non-specific nChap (0.5 mg mL^{-1}) after being stored at 40 ℃ for different times.

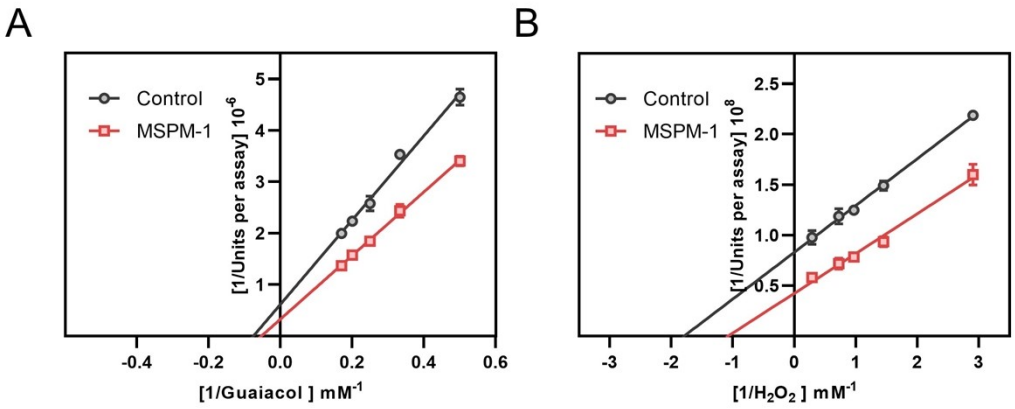

Figure S14. Lineweaver–Burk plot showing the reaction velocity of free HRP and HRP loaded on MSPM-1 with varying H_2O_2 (a) and guaiacol (b) concentrations.