Supporting Information

Thermo-sensitive polycaprolactone coacervates for preventing protein aggregation under thermal stress

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1 Experimental detail

1.1 Materials

VPyCL and Boc-NIPIL were synthesized according to the literature[1] and placed under an argon atmosphere prior to use. Succinic anhydride and CF_3COOH were purchased from Aladdin reagent and used without further purification. Pyridine was purchased from Adamas. All other chemical reagents and solvents were purchased from Greagent and used without further purification. All proteins were purchased from Sigma-Aldrich, which were placed in a 4 ^oC or -20 ^oC refrigerator.

1.2 General characterization

The ^1H Nuclear Magnetic Resonance (NMR) spectra (400 MHz) in Chloroform-*d* were performed at room temperature on a Bruker NMR spectrometer. The temperature-dependent ^1H NMR measurements (600 MHz) was recorded in deuteroxide (D_2O) with temperature increasing from 25 ^oC to 45 ^oC. The ^{13}C Nuclear Magnetic Resonance (^{13}C NMR) spectra (150 MHz) in Chloroform-*d* was performed at room temperature on a Bruker NMR spectrometer. Transmittance measurement fixed in 500 nm wavelength was measured on a UV-Vis chromatogram analyzer (Shanghai Zhetu Science Apparatus Company Limited) with a thermostat sample holder, and the heating rate was 1 °C/min, sample was set in 10 mg/ml concentration. Both the molecular weight and molecular weight distribution were determined by Gel Permeation Chromatography (GPC) on a USA Waters 1515 HPLC using DMF containing 0.01 M LiBr as eluent at a flow rate of 1.0 mL/min. Dynamic light scattering (DLS) was performed on a Malvern Nano-ZS ZEN 3600 apparatus to monitor the zeta-potential at 40 ^oC; the balance time is set to 5 min.

1.3 Synthesis of P(VPyCL-Boc-NIPIL)

Representative $P(VPyCL_{90\%}\text{-}Boc\text{-}NIPIL_{10\%})_{100}$ was synthesized through ring-opening polymerization (ROP) with BnOH as initiator and TBD as a catalyst. A detail procedure was described as follows. 300 mg VPyCL (1.42 mmol, 90 eq) and 34.0 mg Boc-NIPIL (0.158 mmol, 10 eq) were added into the flamed-dried flask with argon protection. And then reaction mixtures were vacuumized for 3 h in 50 ^{o}C oil bath pan. After 3 h, 1.5 mL dichloromethane (DCM) and 28.1 μL benzyl alcohol (BnOH) solution (0.0158 mmol, 18.018 μg/mg in toluene) were added into flash. To this clear solution, 10 mg TBD (5 mol% relative to the monomer molar) was added to catalyze ROP of monomer. After a pre-determined reaction time, extra acetic acid (50 μL) was added to quenched reaction. The polymer was purified through reprecipitation in anhydrous ether or methyl tert-butyl ether three times and dried in vacuum. Finally, 294 mg purified ($P(VPyCL_{90\%}\text{-}PIL_{10\%})_{100}$) (88.0% yield) was collected to further characterize.

1.4 Synthesis of deprotection of P(VPyCL-Boc-NIPIL)

200 mg representative $P(VPyCL_{90\%}\text{-}\ PIL_{10\%})_{100}$ was dissolved in 1 mL dry CH_2Cl_2 and added into the flamed-dried flask with argon protection. Then 500 μL dry CF_3COOH was added. After stirring for 5 h, the deprotected polymer was purified through reprecipitation in anhydrous ether or methyl tert-butyl ether three times and dried in vacuum. Finally, 154 mg purified polymer (81.1% yield) was collected to further characterize.

1.5 Synthesis of post-modification of P(VPyCL-CCL)

To gain carboxylic polymer, succinic anhydride was used to react with P(VPyCL-CCL). A detail procedure was described as follows. 200 mg representative $P(VPyCL_{90\%}\text{-}\ PIL_{10\%})_{100}$ and 20 mg succinic anhydride were added into dry flash. 2 mL dry THF and 100 µL pyridine were then added into the system. After stirring for 24 h at 60 ^{o}C, the mixture was precipitated in anhydrous ether or methyl tert-butyl ether third times and then dried in vacuum. Finally, 165 mg purified polymer (81.1% yield) was collected to further characterize.

Other samples were prepared using a similar procedure.

1.6 Measurement of water content of coacervate or globule

1 mL CPCL-1, CPCL-2 and $PNIPAM_{93}$ solution (c=10 mg/mL in PBS solution) were respectively added into a 2 mL centrifuge tube. Then, tubes were heated to 40 °C or 85 °C to ensure the formation of coacervate or globule. After incubation for 10 min at 40 °C or 85 °C, coacervate or globular droplets were separated from the media through a high speed constant-temperature centrifugation. The supernatant was removed carefully utilizing a micropipette. Relative water content was determined by calculating ratio between the weight loss of the coacervates after freeze-drying (m_1) and the weight of the coacervates without freeze-drying (m_2).

$$\text{Relative water content (\%)} = m_1/m_2 * 100\%$$

1.7 Measurement of degradability of coacervate-forming CPCLs

500 mg CPCL-1 and CPCL-2 were dispersed in 50 mL PBS solution (pH=7.4, 100 mM PBS solution).100 mg pseudomonas cepacia lipase was added into solution and stirred at 37 °C. In comparison, CPCL solution without lipase was treated in similar condition. 5 mL solution was taken at a pre-determined time to measure LCST of degraded polymer solution. ΔT_{cp} was a different value that T_{cp} of CPCLs in different exposure times at 37 °C subtracted original T_{cp} of polyesters.

$$\Delta T_{cp} = T_{cp} - T_{cp0}$$

(T_{cp} was the phase transition temperature of CPCLs solution taken at pre-determined time. T_{cp0} was the phase transition temperature of CPCLs solution without incubation at 37 °C)

1.8 Adsorption of proteins by CPCLs measured by LSCM

Thermo-sensitive CPCLs solution (500 μL, c=10 mg/mL) was mixed with FITC-labelled lysozyme solution (10 μL, c=10 mg/mL) in 2 mL tube. Then mixed solution was placed at 4 °C

or 40 °C for 30 min. Then, aliquots of mixed solution were spread on microscope slides and imaged by LSCM (Laser scanning confocal microscope).

1.9 Determination of binding efficiency for lysozyme

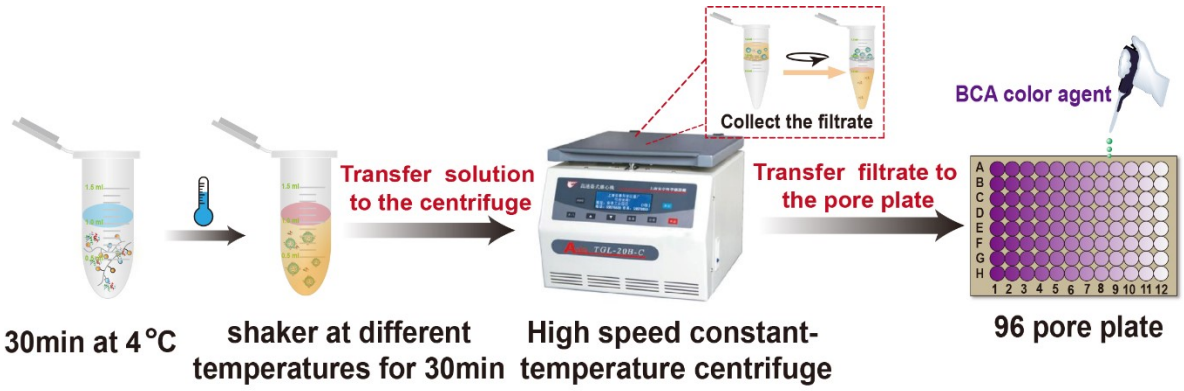


Fig. S1 Schematic diagram of quantifying binding efficiency of polymer for lysozyme.

Binding efficiency for lysozyme was determined according to the bicinchoninic acid (BCA) assay.[2] A detailed procedure was followed. 500 μL thermo-sensitive CPCLs solution (c=10 mg/ml) was mixed with 20 μL protein solution (c=5 mg/ml) in 2 ml tube. The tube containing CPCLs/lysozyme mixed solution was placed at 4 °C fridge for 30 min to ensure sufficient mixture between CPCLs and protein. Then the CPCLs/lysozyme mixture was placed water bath pot with different representative temperatures (4 °C, 25 °C and 40 °C). After 30 min incubation, CPCLs/lysozyme was transferred to a centrifuge filter tube ($M_{co}=10^5$ cutoff). The tube was centrifuged at different representative temperatures for 30 min. In order to determine binding efficiency of lysozyme, 20 μL filtrate was completely mixed with 180 μL BCA chromogenic agent and incubated at 37 °C for 30 min. Then, mixed solution was cooled to room temperature to wait for. The optical density (OD) of solution at 562 nm on a Biotek Synergy H1 multimode plate reader was recorded. PBS and BCA chromogenic agent mixture solution was regarded as negative control. Quantitative protein and BCA chromogenic agent mixture solution were positive controls. Binding efficiency (%) was calculated as follows.

$$\text{Binding efficiency (\%)} = (A_{562nm}^{sample} - A_{562nm}^{negative}) / (A_{562nm}^{positive} - A_{562nm}^{negative}) \times 100\%$$

1.10 Evaluation of protective capability for lysozyme against thermal stress

Heat treatment of lysozyme

500 μL additive solution (c=10 mg/mL) (additives such as CPCLs, $PNIPAM_{93}$, PEG_{113}, betaine, tween 80, sucrose, trehalose, or sorbitol) was mixed with 20 μL lysozyme solution (c=5 mg/mL) in 2 mL tube. The tube containing additive/protein mixed solution was placed at 4 °C fridge for 30 min to ensure sufficient mixture between polymer and protein. Then the tube was kept in an 85 °C water bath pot for 30 min. After 30 min, the tube was immediately taken out to place at 4 °C fridge for 30 min to recover activity. 500 μL PBS solution without additive solution as positive control was mixed with 20 μL lysozyme solution (c=10 mg/mL) in 2 mL tube. Subsequent operations are similar.

Fig. S2 Experimental diagram of relative activity measurement of heat-treated lysozyme

Measurement of lysozyme relative activity

Relative activity of lysozyme was determined according to the colorimetric method.[3] A detailed procedure was followed. 160 μL lysozyme solution (c=192 μg/mL) was added into cuvette containing 2500 μL *Micrococcus lysodeikticus* solution. The optical density (OD) of solution at 450 nm was recorded every 5 sec for 100 sec. The strating OD was set in 0.7~0.9. Relative activity (%) was calculated according to the formula: relative activity (%)=$(\Delta A_{450nm}^{sample}/\Delta A_{450nm}^{PBS}))\times 100\%$ ($\Delta A_{450nm}^{sample}$ was OD change of heated lysozyme during 100 sec, ΔA_{450nm}^{PBS} was OD change of unheated lysozyme). Relative activity of unheated lysozyme was defined as 100%.

1.11 Evaluation of protective capability for acidic and basic proteins against thermal stress

Heat treatment of acidic and basic proteins

Acidic and basic proteins included superoxide dismutase, urease, catalase, horseradish peroxidase, and RNase A. These acidic and basic proteins were repectively mixed with CPCL-1, CPCL-2 and $PNIPAM_{93}$, And then these proteins were thermally treated as lysozyme did.

Measurements of acidic and basic protein relative activity

Superoxide Dismutase (SOD): Before the test, 50 mM pyrogallol in 10 mM HCl solution and NaOH solution (pH 9.0) were prepared, fresh SOD solution, heated-treat SOD solution and heated-treat SOD solution containing additives were prepared according to S1.9. Relative activity of SOD was performed using an improved pyrogallol autoxidation method.[4] The detailed operation was as follows: 100 μL enzyme solution (c=192 μg/mL) was mixed with 100 μL pyrogallol solution. Then, 200 μL mixed solution was added into 2500 μL NaOH solution. The change of absorbance at 325 nm was recorded during 100 s. Additionally, 100 μL PBS solution was used to replace enzyme solution to measure the change of absorbance as a control sample. Relative activity (%) was calculated according to the formula: relative activity (%)=(ΔA_{325nm}^{PBS}-$\Delta A_{325nm}^{sample}$)/$\Delta A_{325nm}^{PBS}$×100% ($\Delta A_{325nm}^{sample}$ was OD change of heated SOD during 100 sec, ΔA_{320nm}^{PBS} was SOD change of unheated SOD). Relative activity of unheated SOD was defined as 100%.

Urease: Before the test, a mixed solution (pH=5.8) containing bromocresol purple (0.015 mM), urea (25 mM), and EDTA (0.2 mM) was prepared. Fresh urease solution, heated-treat urease solution, and heated-treat urease solution containing additives were prepared according to S1.9. Relative activity of urease was performed by monitoring the absorbance change of the pH-sensitive dye bromocresol purple.[4] The detailed operation was as follows: 100 μL enzyme solution (c=192 μg/mL) was added into 2500 μL bromocresol purple solution. Then, mixed solution was placed at 37 °C shaker for 30 min. Absorbance at 588 nm was recorded. Relative

activity (%) was calculated according to the formula: relative activity (%)=(ΔA_{588nm}^{PBS} - $\Delta A_{588nm}^{sample}$)/$\Delta A_{588nm}^{PBS}$×100% ($\Delta A_{588nm}^{sample}$ was absorbance change of heated-treat urease in 37 °C shaker for 30 min, ΔA_{588nm}^{PBS} was absorbance change of unheated-treat urease in 37 °C shaker for 30 min). Relative activity of unheated-treat urease SOD was defined as 100%.

Catalase: Before the test, 100 mM H_2O_2 solution was prepared. Fresh catalase solution, heated-treat catalase solution and heated-treat catalase solution containing additives were prepared according to S1.9. Relative activity of catalase was performed by monitoring the absorbance change of the H_2O_2 solution. The detailed operation was as follows: 200 μL enzyme solution (c=192 μg/mL) was added into 2500 μL the H_2O_2 solution. The change of absorbance at 240 nm was recorded during 100s. Relative activity (%) was calculated according to the formula: relative activity (%)=(ΔA_{240nm}^{PBS} -$\Delta A_{240nm}^{sample}$)/$\Delta A_{240nm}^{PBS}$×100% ($\Delta A_{240nm}^{sample}$ was absorbance change of heated-treat catalase, ΔA_{240nm}^{PBS} was absorbance change of unheated-treat catalase). Relative activity of unheated-treat catalase was defined as 100%.

Horseradish Peroxidase (HRP): Before the test, a mixed solution containing phenol (10 mM), H_2O_2 (0.2 mM) and 4-aminoantipyrin (2.4 mM) was prepared. Fresh HRP solution, heated-treat HRP solution and heated-treat HRP solution containing additives were prepared according to S1.9. Relative activity of HRP was performed by monitoring the absorbance change of the 4-aminoantipyrin solution.[4] The detailed operation was as follows: 10 μL HRP solution (c=48 μg/mL) was added into 2500 μL the mixed solution. The change of absorbance at 510 nm was recorded during 100 s. Relative activity (%) was calculated according to the formula: relative activity (%) = (ΔA_{510nm}^{PBS} -$\Delta A_{510nm}^{sample}$)/$\Delta A_{510nm}^{PBS}$×100% ($\Delta A_{510nm}^{sample}$ was absorbance change of heated-treat HRP, ΔA_{510nm}^{PBS} was absorbance change of unheated-treat HRP). Relative activity of unheated-treat HRP was defined as 100%.

RNase A: Before the test, yeast RNA solution (25 mg/mL) was prepared. Fresh RNase A solution, heated-treat RNase A solution, and heated-treat RNase A solution containing additives

were prepared according to S1.9. Relative activity of RNase A was performed by monitoring the absorbance change of the yeast RNA solution.[4] The detailed operation was as follows: 200 μL RNase A solution (c=200 μg/mL) was added into 2500 μL the mixed solution. Then, mixed solution was placed in 37 ^{o}C shaker for 30 min. The change of absorbance at 260 nm was recorded. Relative activity (%) was calculated according to the formula: relative activity (%) = $(\Delta A_{260nm}{}^{PBS}-\Delta A_{260nm}{}^{sample})/\Delta A_{260nm}{}^{PBS}\times 100\%$ ($\Delta A_{260nm}{}^{sample}$ was absorbance change of heated-treat RNase A at 37 ^{o}C shaker for 30 min, $\Delta A_{260nm}{}^{PBS}$ was absorbance change of unheated-treat RNase A at 37 ^{o}C shaker for 30 min). Relative activity of unheated-treat RNase A was defined as 100%.

1.12 D_h of lysozyme characterized by DLS

D_h of lysozyme was characterized by DLS with a laser light scattering spectrometer (BI-200SM) equipped with a digital correlator (BI-9000AT) at 636 nm. To reduce effect of lysozyme aggregation and additive, the sample was diluted with water to the lysozyme concentration of 10 μg/mL. Then, a diluted sample was measured by DLS at 25 ^{o}C.

1.13 Cytotoxicity assay

Cell viability of CPCL-1 and CPCL-2 and their degraded products (degraded products was purified through filtration (M_{co}=10^4 cutoff) to remove lipase) was measured through MTT assay toward mouse L929 fibroblast cells. Before cell viability experiments, all samples were dissolved in PBS solution with 10mg/ml concentration and sterilized by ultraviolet light for 30 min. Then L929 cells were left to adhere and proliferate on the wells for 24 h and then incubated with a concentration range (from 0 to 1000 μg/mL) of CPCL-1 and CPCL-2 and their degraded products. After incubation for 24 h, MTT solution was added to culture for an additional 4 h, and the average optical density (OD) were measured by Universal Microplate Spectrophotometer. Cell viability (%) was calculated according to the following formula: Cell viability (%) = $(OD_{sample})/(OD_{control})\times 100\%$. Experiments were performed in triplicate.

1.14 Hemolytic test

The hemolytic test on polymer was conducted using rabbit red blood cells (hRBCs). Fresh rabbit blood was washed with PBS solution three times. And then was diluted to a 5% red blood cell suspension. 20 μL diluted polymer solution including CPCL-1 and CPCL-2 and their degraded products (degraded products were purified through filtration (M_{co}=10^4 cutoff) to remove lipase) solution was 2 mL plastic tube and 200 μL blood suspension was added to the plate. Polymer concentration was set from 15.6 to 2000 μg/mL in tube. Then the tube was incubated at 37 °C for 1 hour. After the tube was centrifuged for 10 min, 200 μL supernatant was carefully transferred to 96-well plate, the OD value was recorded at 405 nm on a microplate reader. The hemolysis rate (%) was calculated using (hemolysis rate (%)=(A_{405nm}^{sample}-$A_{405nm}^{negative}$) / ($A_{405nm}^{positive}$-$A_{405nm}^{negative}$)×100%.

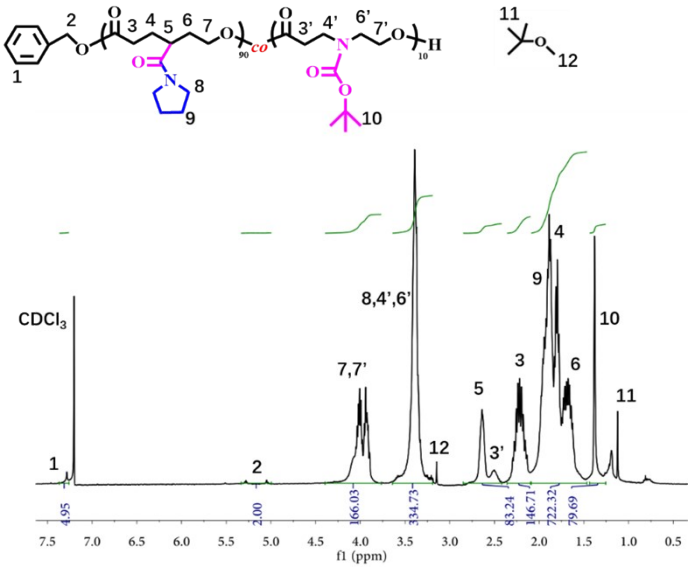


Fig. S3 ^{1}H NMR spectrum of P(VPyCL$_{90\%}$-Boc-NIPIL$_{10\%}$)$_{100}$

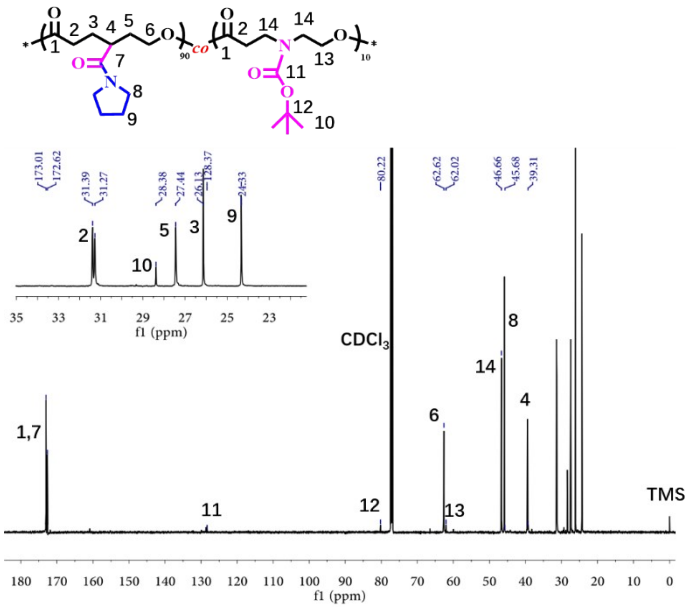
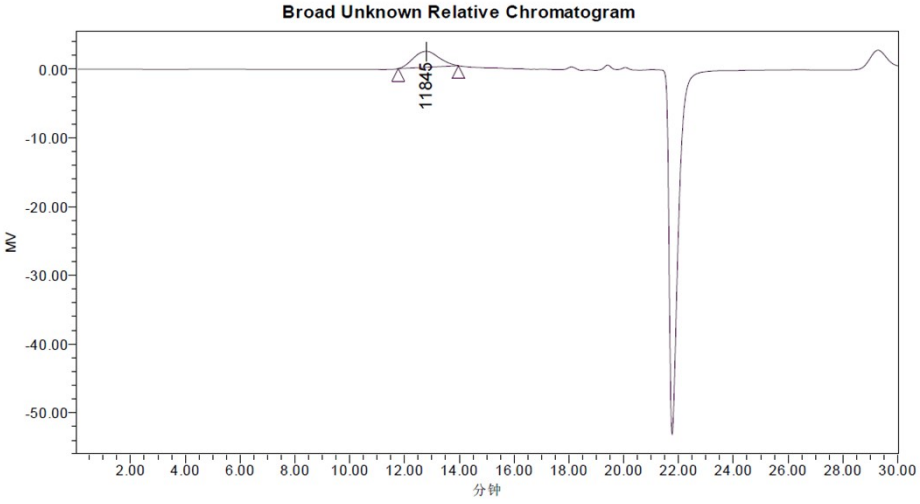


Fig. S4 ^{13}C NMR spectrum of P(VPyCL$_{90\%}$-Boc-NIPIL$_{10\%}$)$_{100}$



Broad Unknown Relative Peak Table

	Distribution Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Mz/Mw	Mz+1/Mw
1		9919	15226	11845	22326	29524	1.535033	1.466241	1.938978

Fig. S5 GPC curve of representative P(VPyCL$_{90\%}$-Boc-NIPIL$_{10\%}$)$_{100}$, M_n=9900, $Đ$=1.53

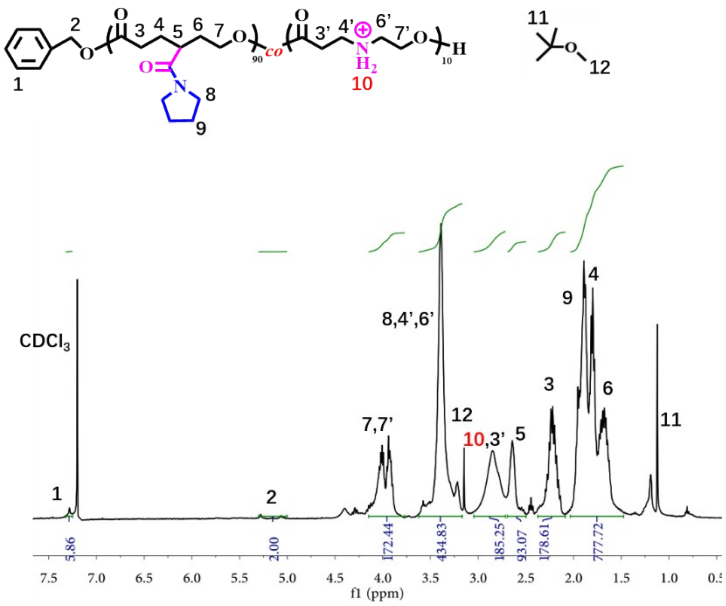


Fig. S6 ^{1}H NMR spectrum of P(VPyCL$_{90\%}$-PIL$_{10\%}$)$_{100}$

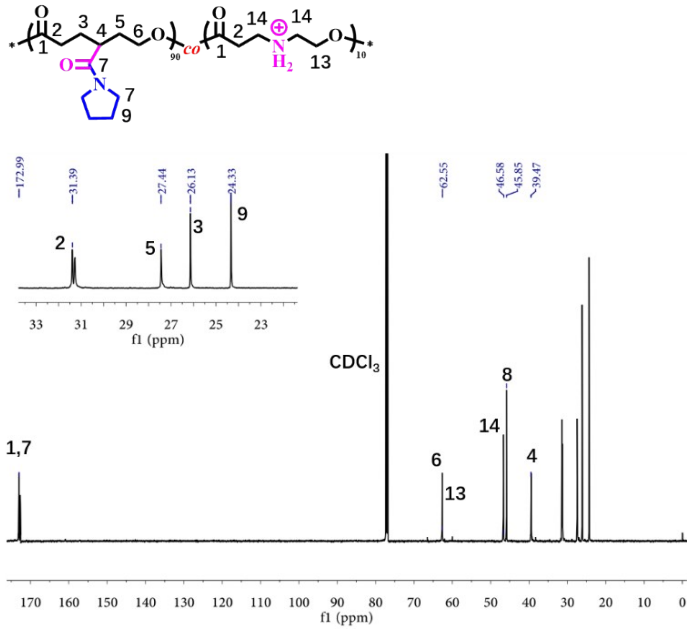


Fig. S7 ^{13}C NMR spectrum of P(VPyCL$_{90\%}$-PIL$_{10\%}$)$_{100}$

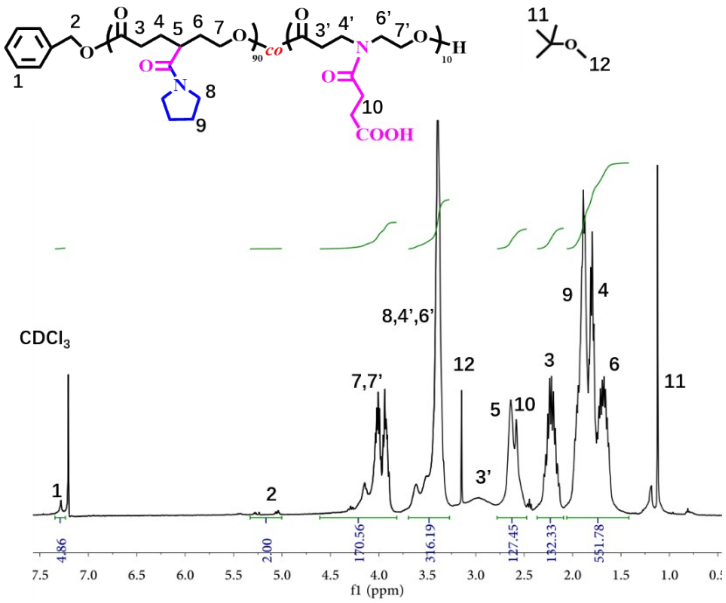


Fig. S8 ^{1}H NMR spectrum of P(VPyCL$_{90\%}$-CCL$_{10\%}$)$_{100}$

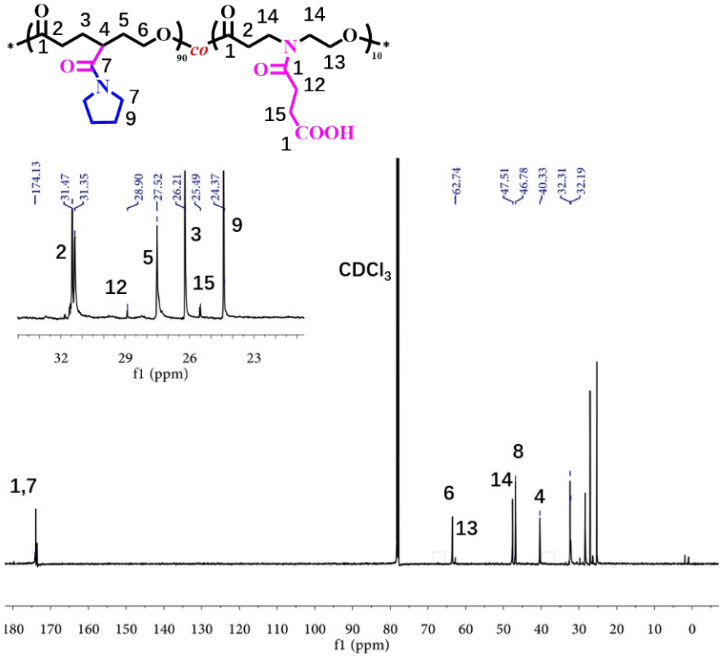


Fig. S9 ^{13}C NMR spectrum of P(VPyCL$_{90\%}$-CCL$_{10\%}$)$_{100}$

Table S1 Characterization results of copolymers

Entry	Polymer	Feed ratio (M_1/M_2)	Composition (M_1/M_2)[a]	Degree of polymerization	M_n/Da [a]	M_n/Da [b]	$Đ$ [b]
1	$PVPyCL_{100}$	100/0	100:0	100	16700	13700	1.26
2	$P(VPyCL_{90\%}$-Boc-$NIPIL_{10\%})_{100}$	90/10	90:10	100	14300	9900	1.53

[a]Composition(M_1/M_2) and number average molecular weight (M_n) were calculated by ^{1}H NMR;

[b]M_n and dispersity ($Đ$) were calculated by GPC.

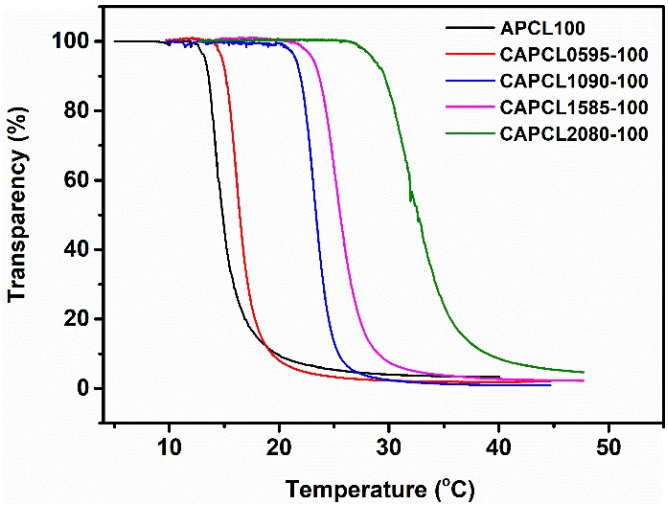


Fig. S10 Change of the transparency vs temperature of APCL100 (CPCL-1 or $PVPyCL_{100}$), CA-PCLs ($P(VPyCL_{x\%}$-Boc-$NIPIL_{y\%})_{100}$) (10 mg/mL in 100 mM PBS solution of pH 7.4).

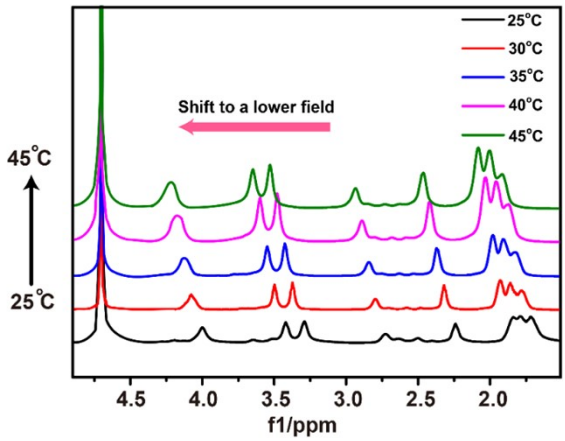


Fig. S11 Variable-temperature ^{1}H NMR of representative CPCL-2 in D_2O

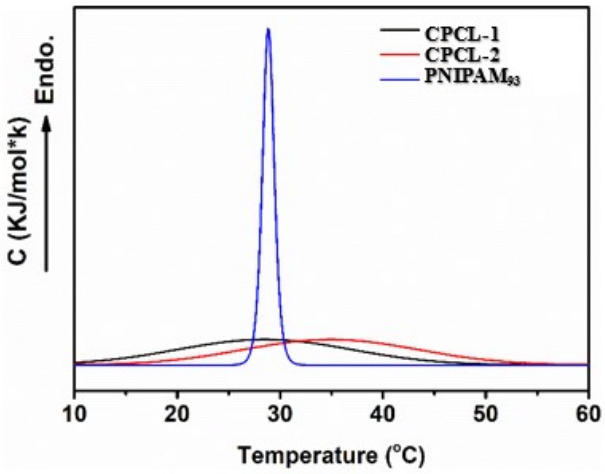


Fig. S12 Fitted thermograms of representative CPCL-1, CPCL-2 and $PNIPAM_{93}$ (10 mg/mL in 100 mM PBS solution of pH7.4) through Gaussian Model measured by Nano-DSC. Scan rate was 1 °C/min

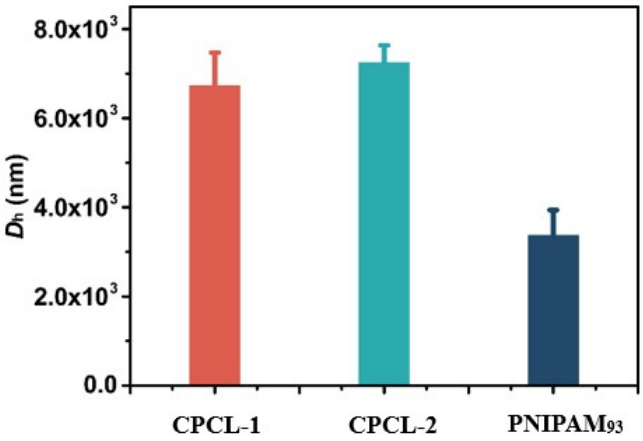


Fig. S13 Hydrodynamic diameter (D_h) of CPCL-1, CPCL-2 coacervate and $PNIPAM_{93}$ aggregation at 85 °C measured by DLS, n=3

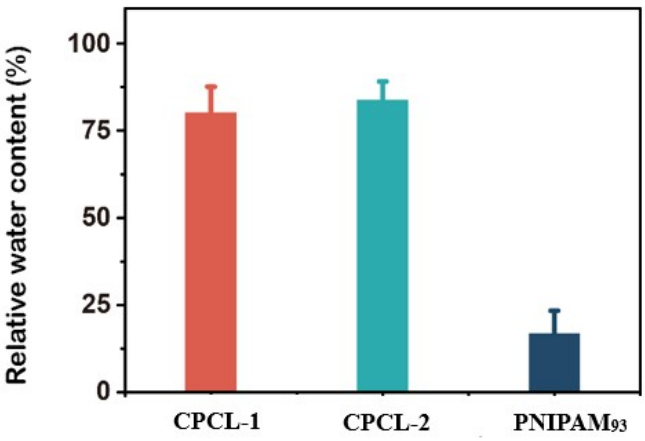


Fig. S14 Relative water content (%) of $PNIPAM_{93,}$ CPCL-1and CPCL-2 at 85 °C, n=3;

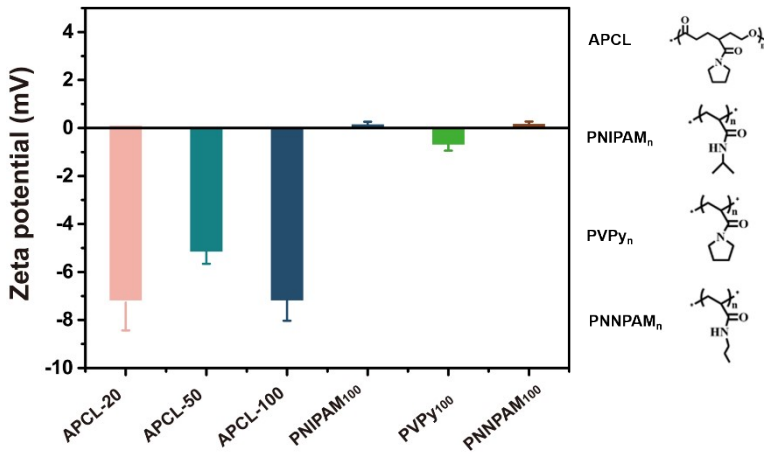


Fig. S15 Zeta potential of different polymers dissolved in pH 7.4 100 mM PBS solution of pH7.4 at 40 °C, *c*=10mg/mL

Table S2 Relative activity of various proteins with different additives

Entry	Protein	M_w/kDa	pI	Relative activity of protein/%			
				No additive	CPCL-1	CPCL-2	$PNIPAM_{93}$
1	Superoxide dismutase (SOD)	32	4.0	54.7±6.6	89.7±14.4	79.1±2.2	70.8±6.4
2	Urease	300	4.8	2.0±0.1	68.4±20.7	37.1±2.7	35.6±2.4
3	Catalase	240	5.4	18.5±3.0	66.2±1.6	60.8±5.5	34.8±0.7
4	Horseradish peroxidase (HRP)	40	7.2	37.0±3.8	85.9±5.5	87.5±3.0	0.9±1.7
5	Lysozyme	14	9.3	2.2±0.7	76.6± 1.3	83.0±4.1	15.0±3.1
6	RNase A	137	9.5	33.9±5.8	84.1±6.2	93.4±2.5	62.2±2.3

[a] Relative activity of all proteins were measured after 85 °C treatment for 30 min. Activity of unheated-treated protein was defined as 100%.

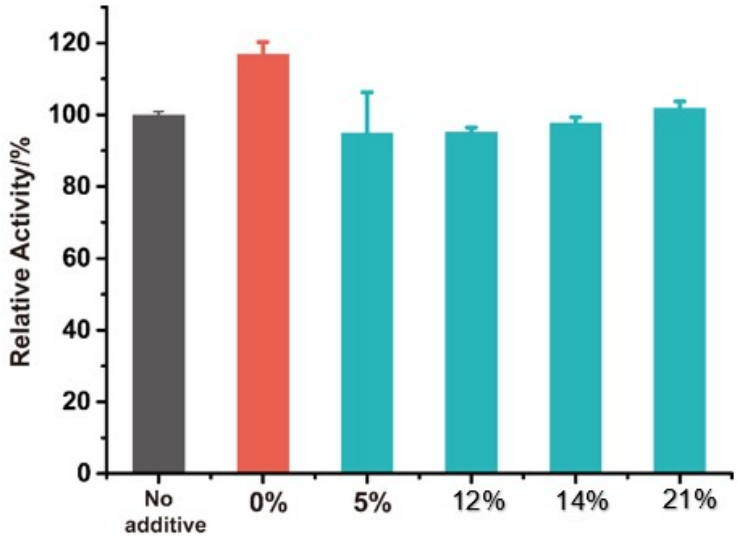


Fig. S16 Relative activity of lysozyme containing CA-PCLs ($P(VPyCL_{y\%}\text{-Boc-NIPIL}_{x\%})_{100}$,) with varied carboxyl substituent contents (x axis) without thermal treatment

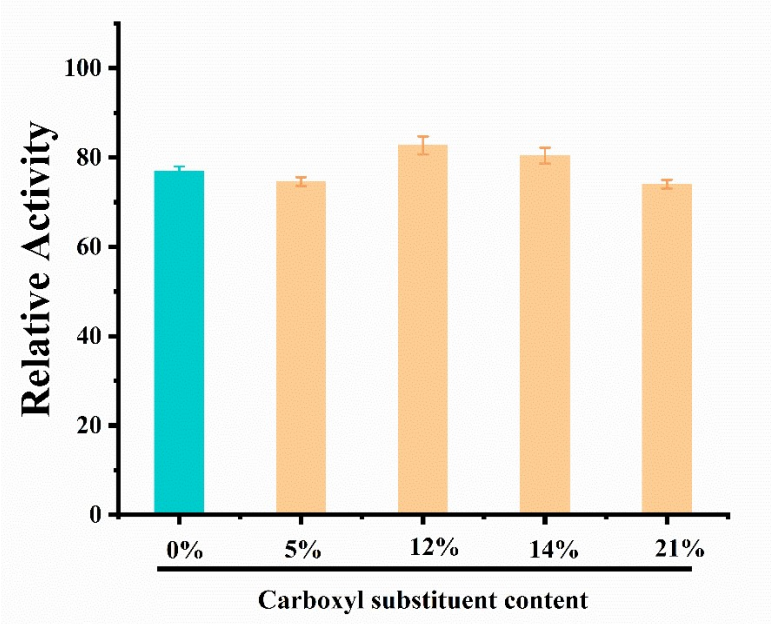


Fig. S17 Relative activity of lysozyme containing CA-PCLs ($P(VPyCL_{y\%}\text{-Boc-NIPIL}_{x\%})_{100}$) with varied carboxyl substituent contents (x axis) after 85 ^{o}C treatment for 30 min

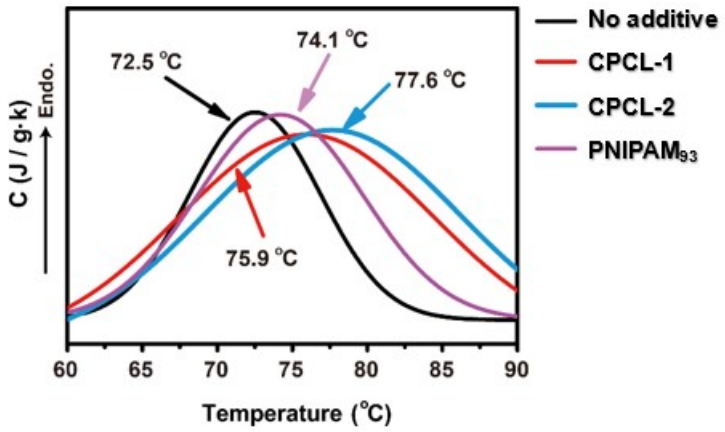


Fig. S18 Fitted thermograms of lysozyme with no additive, CPCL-1, CPCL-2 and $PNIPAM_{93}$ ($c_{polymer}$=10 mg/mL) through Gaussian Model measured by Nano-DSC

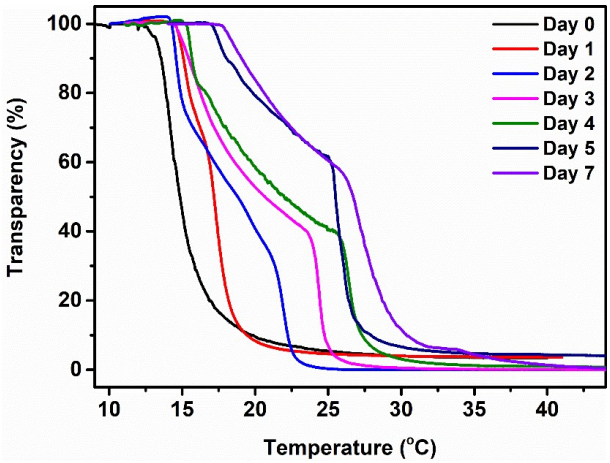


Fig. S19 Change of the transparency *versus* temperature of CPCL-1 exposed at 37 °C in 100 mM PBS solution of pH 7.4.

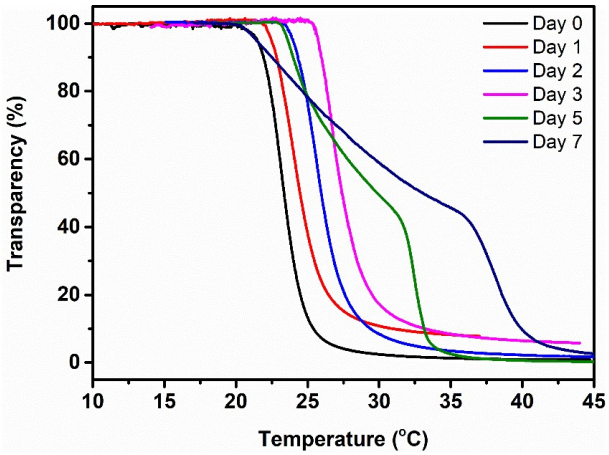


Fig. S20 Change of the transparency *versus* temperature of CPCL-2 exposed at 37 °C in 100 mM PBS solution of pH 7.4.

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