Supplementary Information

Facile and controlled synthesis of poly(ethylene glycol)-polypeptide in an acidic, biphasic system

Jing Huang,[1,5]‡ *Xingliang Liu,*[1,6]‡ *Zhen Yuan,*[1] *Tianjian Yang,*[4] *Yu Zhao,*[2] *Yao Lin,*[4,]* *Ziyuan Song,*[2,]* *Jianjun Cheng,*[1,3,]*

[1]School of Engineering, Research Center for Industries of the Future, Westlake University, Hangzhou, Zhejiang 310030, China

[2]Institute of Functional Nano & Soft Materials (FUNSOM), Jiangsu Key Laboratory for Carbon-Based Functional Materials and Devices, Soochow University, Suzhou 215123, China

[3]Institute of Advanced Technology, Westlake Institute for Advanced Study, Hangzhou, Zhejiang 310024, China

[4]Polymer Program, Institute of Materials Science, Department of Chemistry, University of Connecticut, Storrs, Connecticut 06269, United States

[5]School of Biomedical Engineering and Informatics, Nanjing Medical University, Nanjing, 211100, Jiangsu, China

[6]Department of Biomedical Engineering, School of Engineering, China Pharmaceutical University, Nanjing, 211198, Jiangsu, China

*Corresponding email: chengjianjun@westlake.edu.cn; zysong@suda.edu.cn; yao.lin@uconn.edu

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Materials

All commercial reagents were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and used as received unless otherwise specified. Amino acids were purchased from Tokyo Chemical Industry (Shanghai, China). Triphosgene was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). The initiator poly(ethylene glycol) amine PEG-NH_2 (M_n = 2000) was purchased from SINOPEG Biotechnology Co., Ltd. (Xiamen, China); PEG-NH_2 (M_n = 550 and 5000) were purchased from Laysan Bio, Inc. (Arab, USA); oligomeric (ethylene glycol) amines ($(OEG)_n$-NH_2, n = 2, 3 and 4) and their azide-modified analogues (N_3-$(OEG)_n$-NH_2, n = 2 and 3) were purchased from Bide Pharmatech Co., Ltd. (Shanghai, China). Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Monomers including *γ*-benzyl-L-glutamate *N*-carboxyanhydride (BLG-NCA), *γ*-(4-propargyloxy)benzyl-L-glutamate (POB)-NCA, N^{ε}-carboxybenzyl-L-lysine (ZLL)-NCA and *γ*-Ethyl-L-glutamate (ELG)-NCA were synthesized following literature procedures.[1] Aqueous BCP buffer with various pH values were prepared according to previous literature procedures,[2] which contains H_3BO_3, citric acid, and H_3PO_4 as active components.

Instruments

^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were collected on a 600 MHz-Solution NMR Spectrometer, Westlake University. Chemical shifts (*δ*) were reported in ppm and referenced to the residual protons in deuterated solvents. MestReNova software (version 14.0.0, Mestrelab Research, Escondido, CA, USA) was used for all NMR analysis. Fourier transform infrared (FTIR) spectra were recorded on a Perkin Elmer 100 serial FTIR spectrophotometer (PerkinElmer, Santa Clara, CA, USA) calibrated with polystyrene film. Gel permeation chromatography (GPC) experiments were performed on a system equipped with an isocratic pump (1260 Infinity II, Agilent, Santa Clara, CA, USA), a multi-angle static light scattering (MALS) detector (DAWN HELEOS-II, Wyatt Technology, Santa Barbara, CA, USA), and a differential refractometer (dRI) detector (Optilab, Wyatt Technology, Santa Barbara, CA, USA). The detection wavelength of HELEOS was set at 658 nm. Separations were performed using serially connected size exclusion columns (three Shodex packed columns KD-803, KD-804 and KD-805, 10 μm, 8 × 300 mm, Yokohama, Japan) at 50 °C. *N*,*N*-dimethylformamide (DMF) containing LiBr (0.1 mol/L) was used as the mobile phase. The MALS detector was calibrated using pure toluene and can be used for the determination of the absolute molecular weights (MWs). The MWs of polymers were determined based on the d*n*/d*c* value of each polymer sample based on the internal calibration system processed by the ASTRA 8.1.2 software (version 8.12, Wyatt Technology, Santa Barbara, CA, USA). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were recorded on a Bruker Rapiflex in the mass spectrometry laboratory, Westlake University, using *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB) as the matrix.

Polymerization of purified NCA in acidic biphasic system

Polymerization of purified NCA in acidic biphasic system was carried out under ambient conditions. Typically, BLG-NCA (10 mg, 0.038mmol) was dissolved in 0.38 mL chloroform. Then, the chloroform solution of TMA (0.5 M, 22.8 μL, 1.14 mmol) and 0.1 mL BCP buffer (pH = 3.0) were added into the above solution, sequentially. Finally, the chloroform solution of PEG-NH_2 (3.8 mM, 100 μL, 0.38 μmol) was added into the above mixture under stirring to start the polymerization, with final chloroform:water = 5:1 (v/v).

For the polymerization at $[M]_0/[I]_0 > 100$, the aqueous pH was adjusted to 7 after 40 min of polymerization, which further accelerated the polymerization kinetics and minimized the side reactions.

When > 99% of NCA monomers were consumed as monitored by FTIR, the organic phase was dropped into hexane:ether (1:1, v/v). The white precipitate was then centrifugated and dried under vacuum, yielding the resulting PEG-polypeptides. The obtained PEG-polypeptides were dissolved in DMF containing 0.1 M LiBr and analyzed by GPC after filtration by a 0.22 μm PTFE membrane (Thermo Fisher Scientific, Waltham, MA, USA).

Polymerization with other initiators, monomers and acids were conducted in a similar way.

Polymerization kinetics

In a typical FTIR experiment, an aliquot of reaction mixture (~ 2.5 μL) was taken out at different time intervals and dried on a KBr salt plate for FTIR characterization, until the disappearance of anhydride peaks from NCA at 1860 cm^{-1} and 1790 cm^{-1}. The concentration of NCA monomers was then quantified through the standard curve based on the absorbance at 1790 cm^{-1}.

The partition of organic acid in a biphasic system

The biphasic partition of organic acid was quantified to study the impact of acid structure on the polymerization behavior. Typically, organic acid (2 M, 29.7 μL, 5.94×10^{-5} μmol) was added into $CDCl_3$ (2.5 mL), followed by the addition of D_2O (0.5 mL). The above mixture was vortexed for 10 s. After phase separation, The $CDCl_3$ phase (0.5 mL) and the aqueous phase (0.3 mL) were carefully taken out and mixed with 2.5 and 1 μL DMSO-d_6 solution (internal standard), respectively. The amount of acid in organic phase and aqueous phase was determined *via* the ratio of the integral of acid protons to DMSO protons.

Water-induced hydrolysis of NCA monomers

Purified BLG-NCAs were dissolved in chloroform containing BCP buffer (50 mM) with various pH values, and the resulting biphasic mixture was vigorously stirred at room temperature. At different time intervals, an aliquot of mixture (~ 2.5 μL) was taken out, dried on a KBr salt plate, and characterized by FTIR. The decrease in anhydride peaks (1860 and 1790 cm^{-1}) from NCAs and the increase in amide peaks (1652 cm^{-1}) from oligopeptides/polypeptides evidenced the oligomerization/polymerization of NCAs.

Synthesis of crude NCA monomers

To a pressure vessel with a heavy wall, γ-benzyl-L-glutamate (5.0 g, 21.1 mmol), THF (75 mL), methyloxirane (6.5 mL, 84.5 mmol) were added sequentially under magnetic stirring.[1] Triphosgene (3.2 g, 10.5 mmol) was finally added in one portion and the vessel was sealed immediately. The amino acid gradually disappeared in ~30 min with a noticeable heat release. The reaction was stirred at room temperature for ~1.5 h. The reaction mixture was then poured into 500 mL hexane. Crude BLG-NCA was obtained as white precipitate, which was centrifugated and dried under vacuum without any additional purification procedures (yield: 86%). The crude NCA could be stored at -20 °C for at least 1 year without significant degradation.

Crude ZLL-NCA, ELG-NCA and POB-NCA were prepared in similar processes.

NMR titration

NMR titration experiments were conducted to probe the molecular interactions between BLG-NCA and TMA. Briefly, BLG-NCA (10.00 mg, 0.038 mmol) was dissolved in CD_2Cl_2 (380 μL), into which various amounts of TMA were added, so that the [TMA]/[BLG-NCA] ratio varied from 0.01 to 0.6. ^{1}H NMR spectra and chemical shifts of ring -NH- at different [TMA]/[BLG-NCA] ratios were recorded.

Supplementary Figures

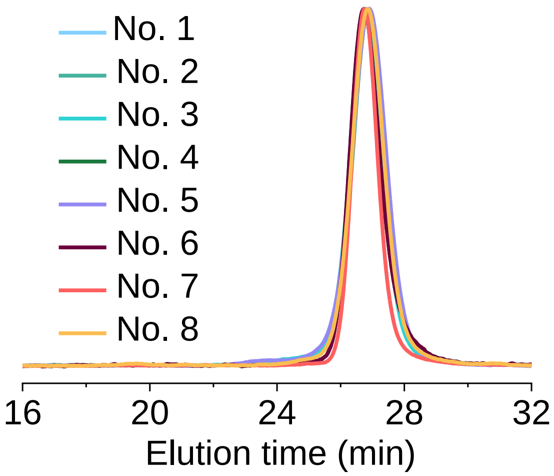


Figure S1. GPC-LS traces of the resulting PEG-PBLG initiated by PEG-NH_2 in the presence of TMA in an acidic, biphasic system. The polymerization was repeated 8 times. $[M]_0$ = 0.1 M, $[M]_0/[I]_0/[\text{TMA}]_0$ = 100/1/30, aqueous pH = 3.0, chloroform/water = 5:1 (v/v).

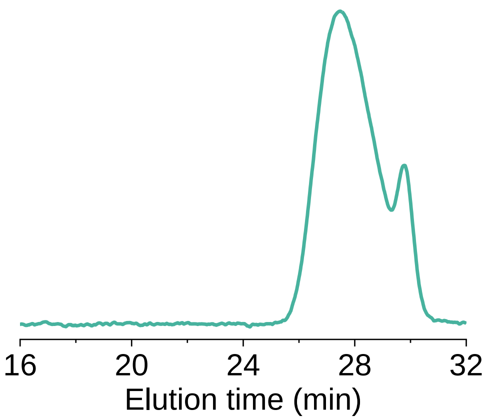


Figure S2. GPC-LS traces of the resulting polypeptides initiated by PEG-NH_2 in the absence of TMA in an acidic, biphasic system. $[M]_0 = 0.1$ M, $[M]_0/[I]_0 = 10$, aqueous pH = 3.0, chloroform/water = 5:1 (v/v).

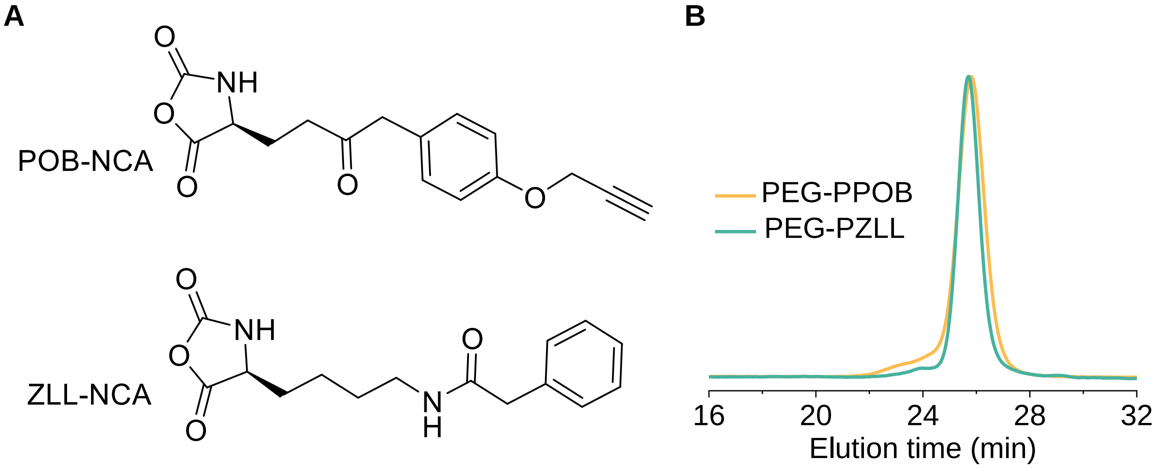


Figure S3. (A) Chemical structures of POB-NCA and ZLL-NCA. (B) Normalized GPC-LS traces of the resulting polypeptides initiated by PEG-NH_2 from POB-NCA and ZLL-NCA in an acidic, biphasic system. $[M]_0$ = 0.1 M, $[M]_0/[I]_0/[\mathrm{TMA}]_0$ = 100/1/30, aqueous pH = 3.0, chloroform/water = 5:1 (v/v).

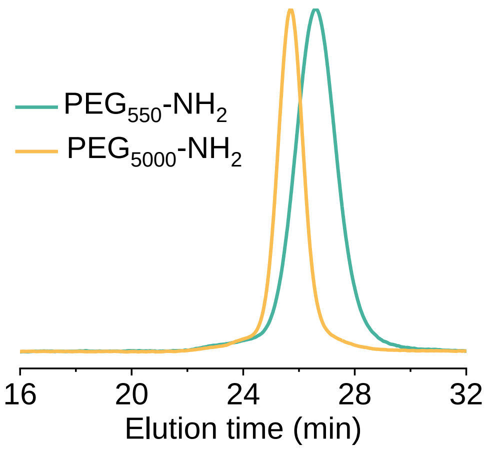


Figure S4. Normalized GPC-LS traces of the resulting polypeptides initiated by PEG-NH$_2$ with MWs of 550 and 5000 Da in an acidic, biphasic system. $[M]_0 = 0.1$ M, $[M]_0/[I]_0/[\mathrm{TMA}]_0 = 100/1/30$, aqueous pH = 3.0, chloroform/water = 5:1 (v/v).

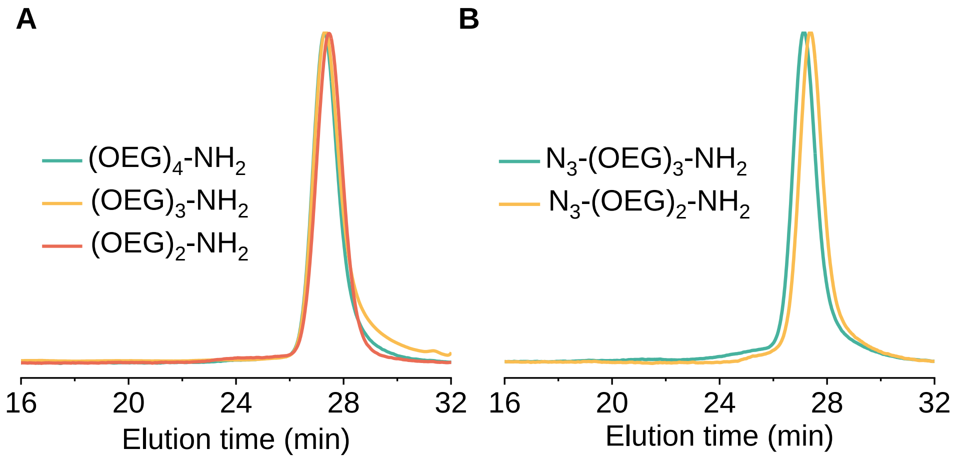


Figure S5. Normalized GPC-LS traces of the resulting polypeptides initiated by (A) $(OEG)_n$-NH_2 and (B) N_3-$(OEG)_n$-NH_2 in the acidic, biphasic system. $[M]_0 = 0.1$ M, $[M]_0/[I]_0/[TMA]_0 = 100/1/30$, aqueous pH = 3.0, chloroform/water = 5:1 (v/v).

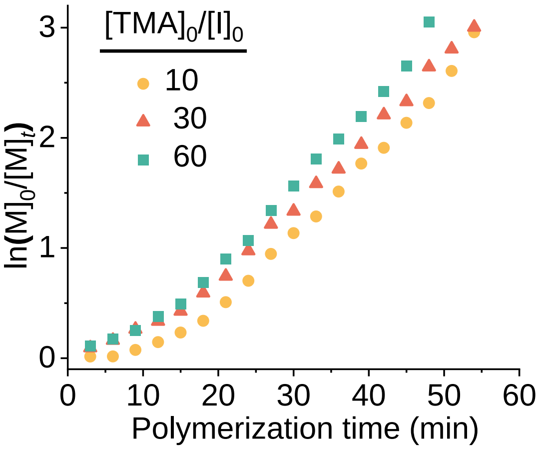


Figure S6. Semilogarithmic kinetic plot of polymerization of BLG-NCA initiated by PEG-NH_2 at various [TMA]$_0$/[I]$_0$ ratios in a biphasic system. $[M]_0 = 0.1$ M, $[M]_0/[I]_0 = 100$, aqueous pH = 3.0, chloroform/water = 5:1 (v/v).

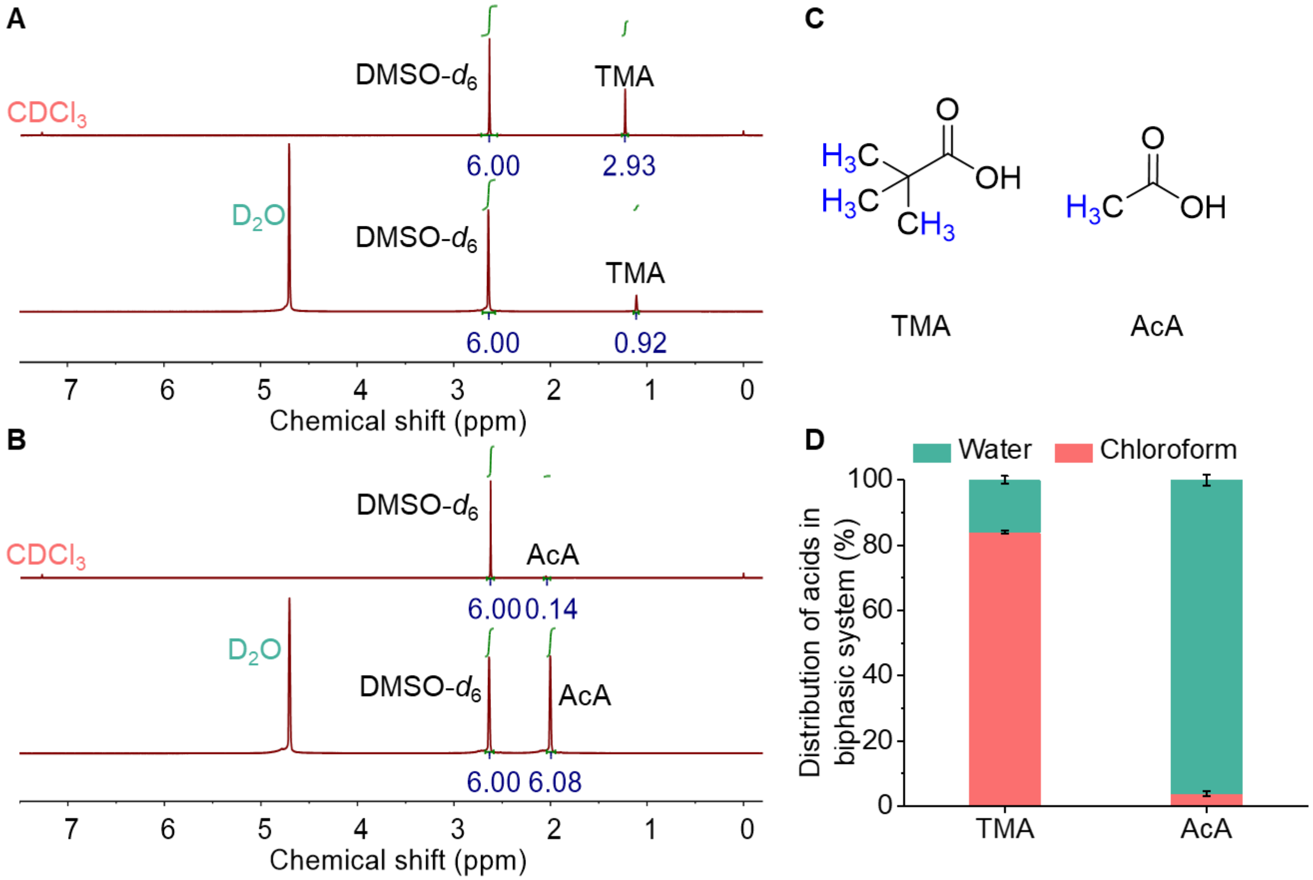


Figure S7. (A) ^{1}H NMR spectra of TMA in organic phase and aqueous phase. (B) ^{1}H NMR spectra of AcA in organic phase and aqueous phase. (C) Chemical structures of TMA and AcA. (D) Distribution of TMA and AcA in a chloroform/water biphasic system. Assuming the volume ratio of water to chloroform is 1:5. 2.5 and 1 μL DMSO-d_6 solution (internal standard) was added into oil and aqueous phase, respectively.

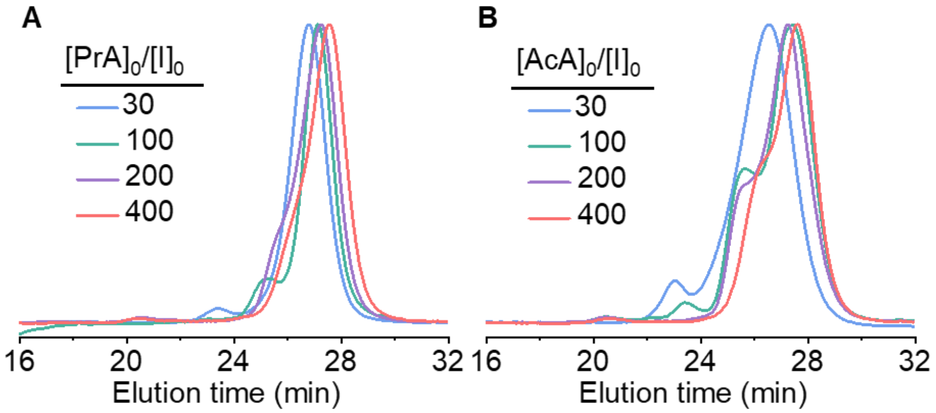


Figure S8. Normalized GPC-LS traces of the obtained PEG-PBLG initiated by PEG-NH_2 at various $[TMA]_0/[I]_0$ ratios in the presence of (A) PrA and (B) AcA. $[M]_0$ = 0.1 M, $[M]_0/[I]_0$= 100, aqueous pH = 3.0, chloroform/water = 5:1 (v/v). AcA = acetic acid; PrA = propionic acid.

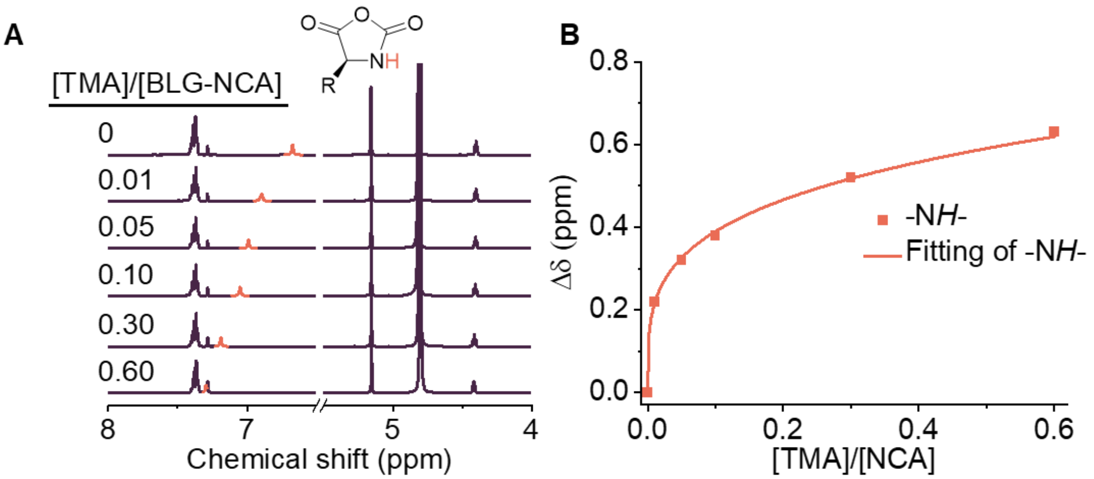


Figure S9. (A) Overlaid ^{1}H NMR spectra of BLG-NCA in biphasic system and (B) The change in chemical shift of -NH- proton in NCA at various [TMA]/[BLG-NCA] ratios. $[M]_0 = 0.1$ M, aqueous pH = 3.0, chloroform/water = 5:1 (v/v).

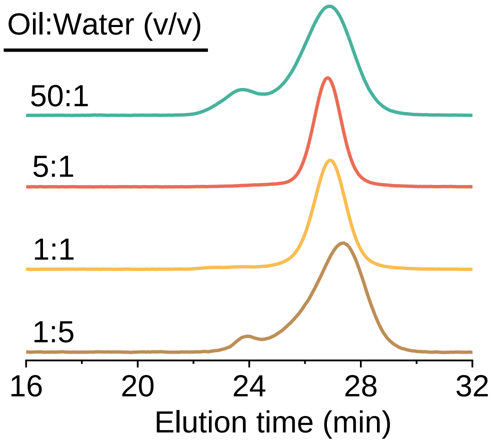
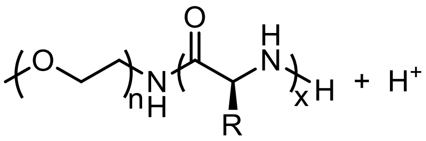


Figure S10. Normalized GPC-LS traces of the resulting PEG-PBLG initiated by PEG-NH2 in a biphasic system with various oil:water ratios. $[M]_0 = 0.1$ M, $[M]_0/[I]_0/[\text{TMA}]_0 = 100{:}1{:}30$, aqueous pH = 3.0.

Supplementary Tables

Table S1. Comparison of Representative *m*/z Signals of the Calculated Values From Molecular Formula and Obtained Values From MALDI-TOF Spectrum.



n	*DP*	Calcd.	Found
53	4	3241.7	3241.6
54	4	3285.7	3285.6
55	4	3329.7	3329.4
56	4	3373.7	3373.1
57	4	3417.8	3417.0
53	5	3460.8	3460.7

The obtained *m*/z signals for PEG-PBLG agree well with the calculated values ($44.026n + 219.09DP$) ($[M + H]^+$), indicating negligible degradation of terminal amino groups.

Table S2. The Acidic, Biphasic Polymerization of BLG-NCA Initiated by PEG-NH_2 in the Presence of Organic Acids.[a]

entry	acid[b]	$[Acid]_0/[I]_0$	t (min)[c]	$M_{n,\,GPC}$ (kDa)[d]	$Đ$[d]
1	TMA	0	60	37.3	1.38
2	TMA	10	54	22.9	1.20
3	TMA	30	55	23.8	1.05
4	TMA	60	48	23.0	1.05
5	AcA	30	48	21.8	1.21
6	PrA	30	50	23.8	1.07
7	IsoBA	30	60	22.4	1.08
8	CyHA	30	70	23.7	1.05

[a] All polymerizations were conducted in an acidic, biphasic system at room temperature. $[M]_0 = 0.1$ M, $[M]_0/[I]_0 = 100$, aqueous pH = 3.0, chloroform/water = 5:1 (v/v). [b] TMA = trimethylacetic acid, AcA = acetic acid, PrA = propanoic acid, IsoBA = isobutyric acid, and CyHA = cyclohexylacetic acid. [c] Polymerization time reaching 95% monomer conversion. [d] Determined by GPC (*N*,*N*-dimethylformamide (DMF) containing 0.1 mol/L LiBr was used as the mobile phase). d*n*/d*c* = 0.104. The theoretical MW for all polymerizations was 23.9 kDa.

Table S3. The Acidic, Biphasic Polymerization of BLG-NCA Initiated by PEG-NH_2 at Various Oil/Water Ratios.[a]

entry	Oil/Water (v/v)	t (min)[b]	$M_{\mathrm{n,GPC}}$ (kDa)[c]	$Đ$[c]
1	50:1	30	21.4	1.21
2	5:1	54	23.8	1.05
3	1:1	51	22.8	1.05
4	1:5	39	17.3	1.21

[a] All polymerizations were conducted in an acidic, biphasic system at room temperature. $[M]_0 = 0.1$ M, $[M]_0/[I]_0/[\mathrm{TMA}]_0 = 100/1/30$, aqueous pH = 3.0. [b] Polymerization time reaching 95% monomer conversion. [c] Determined by GPC (N,N-dimethylformamide (DMF) containing 0.1 mol/L LiBr was used as the mobile phase). dn/dc = 0.104. The theoretical MW for all polymerizations was 23.9 kDa.

Table S4. Polymerization of BLG-NCA Initiated by PEG-NH_2 in Biphasic System with Various Aqueous pH.[a]

entry	pH	t (min)[b]	$M_{n,GPC}$ (kDa)[c]	$Đ$[c]
1	2	90	23.6	1.05
2	3	55	23.7	1.05
3	4	18	23.3	1.23
4	5	18	23.5	1.39
5	6	16	30.0	1.27
6	7	15	34.6	1.27
7	8	15	36.4	1.23

[a] All polymerizations were conducted in an acidic, biphasic system at room temperature. $[M]_0$ = 0.1 M, $[M]_0/[I]_0/[\mathrm{TMA}]_0$= 100/1/30, chloroform/water = 5:1 (v/v). [b] Polymerization time reaching 95% monomer conversion. [c] Determined by GPC (*N*,*N*-dimethylformamide (DMF) containing 0.1 mol/L LiBr was used as the mobile phase). d*n*/d*c* = 0.104. The theoretical MW for all polymerizations was 23.9 kDa.

Table S5. The Synthesis of Multiblock PEG-Copolypeptides Initiated by PEG-NH_2 From Crude Monomers in the Acidic, Biphasic System. [a]

block No.	monomer	$[M]_0/[I]_0$	t (min) [b]	$M_{\text{n, GPC}}$ (kDa)[c]	$M_{\text{n, theo.}}$ (kDa)	$Đ$[c]
1	BLG-NCA	50	35	12.2	12.9	1.05
2	ELG-NCA	50	54	20.2	23.0	1.09
3	ZLL-NCA	50	54	35.9	38.3	1.05
4	POB-NCA	50	45	53.6	53.9	1.05

[a] All polymerizations were conducted in an acidic, biphasic system at room temperature. $[M]_0 = 0.1$ M, $[\text{TMA}]_0/[I]_0 = 30$, chloroform/water = 5:1 (v/v). [b] Polymerization time reaching 95% monomer conversion. [c] Determined by GPC (*N*,*N*-dimethylformamide (DMF) containing 0.1 mol/L LiBr was used as the mobile phase).

Supplementary References

(1) Tian, Z. Y.; Zhang, Z.; Wang, S.; Lu, H. A Moisture-Tolerant Route to Unprotected α/β-Amino Acid *N*-Carboxyanhydrides and Facile Synthesis of Hyperbranched Polypeptides. *Nat. Commun.* **2021**, 12, 5810.

(2) Carmody, W. R. Easily Prepared Wide Range Buffer Series. *J. Chem. Educ.* **1961**, 38, 559.