Supporting Information

Modulation of polymerization rate of *N*-carboxyanhydrides in a biphasic system

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Materials

All chemicals were purchased from Aladdin (Shanghai, China) and used as received unless otherwise specified. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, USA). Tetrahydrofuran (THF), *n*-hexane, diethyl ether, dichloromethane (DCM), chloroform, and *N*,*N*-dimethylformamide (DMF) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Methoxy poly(ethylene glycol) amine (PEG, 5 kDa) was purchased from Laysan Bio Inc. (Arab, USA). Anhydrous THF and hexane was dried in a column using alumina. Poly(amidoamine) dendrimer (PAMAM, generation 3.0) was purchased from MilliporeSigma (St. Louis, USA). The monomer γ-benzyl-L-glutamate *N*-carboxyanhydride (BLG-NCA) and N^{ε}-carboxybenzyl-L-lysine NCA (ZLL-NCA) were synthesized following literature procedures [1]. Aqueous buffer with a wide pH range from 3.0 to 9.0 were prepared according to previous literature reports [2]. The pH values of all aqueous buffers were confirmed with a pH meter, which showed an error of less than 0.2 pH compared to the theoretical value.

Instrumentation

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Varian $^{\text{UNITY}}$INOVA-300 spectrometer, Agilent Direct-Drive II 600 MHz spectrometer, and Bruker Ascend 400 MHz spectrometer in the NMR laboratory in Soochow University. The chemical shifts (δ) were reported in ppm and referenced to the residual protons in the deuterated solvents. MestReNova software (version 6.1.0, Mestrelab Research, Escondido, USA) was used for all NMR analysis. Gel permeation chromatography (GPC) experiments were performed on a system equipped with an isocratic pump (1260 Infinity II, Agilent, Santa Clara, USA), a multi-angle static light scattering (MALS) detector (DAWN, Wyatt Technology, Santa Barbara, USA), and a differential refractometer (dRI) detector (Optilab, Wyatt Technology, Santa Barbara, USA). The detection wavelength of the MALS detector was set at 658 nm. Separations were performed using serially connected size exclusion columns (KD-803, KD-804, and KD-806, 8 × 300 mm, Shodex, Yokohama, Japan) using DMF containing 0.1 mol/L LiBr as the mobile phase at a flow rate of 1 mL/min at 60 °C. 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 calculated offline by using the internal calibration system processed by the ASTRA 8 software (version 8.1.0, Wyatt Technology, Santa Barbara, USA). Fourier transform infrared (FTIR) spectra were recorded using a Thermo Fisher Nicolet iS20 FTIR spectrometer. Dynamic light scattering (DLS) experiments were performed using a Zeta-sizer Nano ZS90 (Malvern Panalytical Ltd, Malvern, UK) with a He-Ne laser (λ = 633 nm) at a scattering angle of 90° at 25 °C. The pH of the aqueous solution was monitored by a pH meter (PHS-3C, Shanghai Leici, Shanghai, China). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were collected on a Bruker ultrafleXtreme in the mass spectrometry laboratory in Soochow University, with *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) as the matrix.

Preparation of PEG-PBLG and PLL macroinitiators

The methoxy poly(ethylene glycol)-*b*-poly(γ-benzyl-L-glutamate) amine (PEG-PBLG) macroinitiators were prepared according to literature procedures, by polymerizing BLG-NCA from a PEG initiator [3]. In a glovebox, PEG (38.0 mg, 0.0076 mmol) and BLG-NCA (60 mg, 0.23 mmol) were dissolved in anhydrous DMF (800 μL) and mixed, and the polymerization was incubated at 4 ºC for 60 h until complete conversion of NCA as monitored by FTIR. After purification by precipitation in cold hexane/ether (1:1, v/v), the obtained PEG-PBLG macroinitiators were re-dissolved in DCM (100 mg/mL) and stored at -30 ºC in a glovebox (Yield: 98%). The stock solution of PEG-PBLG macroinitiators was used within one week to avoid the degradation of terminal amino groups.

The poly(L-lysine) (PLL) macroinitiators were synthesized by the polymerization of ZLL-NCA followed by the deprotection of side chains. In a glovebox, ZLL-NCA (200.0 mg, 0.65 mmol) was dissolved in anhydrous DMF, into which the DMF solution of *n*-hexylamine (0.1 mol/L, 218 μL, 0.0218 mmol, $[M]_0/[I]_0$ = 30) was added. The polymerization was carried out at rt overnight until complete conversion of NCA as monitored by FTIR. The product poly(N^{ε}-carboxybenzyl-L-lysine) (PZLL) was purified by precipitation in hexane/ether (1:1, v/v) and dried under vacuum (Yield: 83%). The obtained PZLL (70 mg, containing 0.27 mmol benzyl groups) was then dissolved in trifluoroacetic acid (TFA) at 0 ºC in an ice bath, into which the HOAc solution of

HBr (33 wt%, 234 μL, 1.33 mmol) was added through a syringe. The solution was stirred at 0 ºC for 2 h. After the removal of solvent and excessive HBr under vacuum, distilled (DI) water (2 mL) was added to dissolve the solid residue. The byproduct benzyl bromide was removed by extraction with ether (3 × 3 mL). The product PLL was purified by dialysis (MWCO = 1 kDa) for 24 h followed by lyophilization (Yield: 88%).

Polymerization kinetics

The polymerization kinetics was monitored in situ by ^{1}H NMR in chlorinated solvents including CD_2Cl_2 or $CDCl_3$. Taking C6-diNH_2-initiated polymerization (C6-diNH_2 initially dissolved in chloroform) as an example, BLG-NCA (13.12 mg, 0.05 mmol) was dissolved in $CDCl_3$ (460 μL, $[M]_0$ = 0.1 mol/L) and mixed with the aqueous buffer (pH 9.0, 14.8 μL, water:$CDCl_3$ = 1:100 (w/w)), into which the $CDCl_3$ solution of C6-diNH_2 (0.05 mol/L, 40 μL, $[M]_0/[I]_0$ = 50) was added to start the polymerization. The mixture was then vortexed and transferred into an NMR tube, and the NMR spectra were monitored at different time intervals. In order to determine the conversion of NCA at different time, the integral of α-H signal of BLG-NCA (δ = 4.36 ppm) was normalized compared to the α-H signal at t = 0 (i.e., 100% remaining NCA). The α-H signal at t = 0 was calculated based on the integral ratios of side-chain benzyl peaks between BLG-NCA (δ = 5.13 ppm) and resulting poly(γ-benzyl-$_L$-glutamate) (PBLG) (δ = 5.08 ppm).

For polymerization kinetics with C6-diNH_2 initially dissolved in aqueous phase, C6-diNH_2 was first dissolved in the aqueous buffer (pH 9.0, 0.3 mol/L), whose pH was measured and re-adjusted with HCl due to the basic nature of the initiator. The aqueous stock solution was then diluted with aqueous buffer (pH 9.0) so that the final concentration of C6-diNH_2 was 0.2 mol/L. The resulting aqueous solution was then mixed with the $CDCl_3$ solution of BLG-NCA (500 μL, $[M]_0$ = 0.1 mol/L, $[M]_0/[I]_0$ = 50, water:$CDCl_3$ = 1:100 (w/w)) to start the polymerization. The polymerization kinetics initiated by C6-diNH_2 at other aqueous pH was conducted in a similar way.

The polymerization kinetics initiated by PAMAM was conducted in a similar way. Due to the rapid kinetics, the kinetics was also monitored by FTIR. Briefly, an aliquot of the polymerization mixture (~10 μL) taken out and added on top of a KBr salt plate, which was dried and checked

by FTIR. The disappearance of anhydride signals from BLG-NCA (1780 and 1850 cm^{-1}) indicated the > 99% conversion of monomer.

The polymerization kinetics initiated by PEG-PBLG was conducted in a similar way, by mixing the CD_2Cl_2 solution of BLG-NCA with CD_2Cl_2/water emulsion of PEG-PBLG. CD_2Cl_2 was used as the solvent to compare the kinetics with previous published results [3].

The degradation kinetics of BLG-NCA was conducted in a similar way, but without the addition of any initiators. While the polymerization mixture in the presence of C6-diNH_2 or PAMAM failed to form a stable emulsion, the water/oil emulsion was stable in the presence of PEG-PBLG for at least 4 h [3]. Therefore, to better mimic the polymerization condition of PEG-PBLG-initiated polymerization, the degradation kinetics was monitored in the presence of mPEG-NHAc, which was prepared by reacting PEG with acetic anhydride [3].

Determination of p*K*a of initiators

The p*K*a values of C6-diNH_2 and PAMAM were determined potentiometrically by a pH meter. Typically, the aqueous solution of PAMAM (10 mg/mL, 400 μL) was mixed with NaCl (1 mol/L, 125 μL) and DI water (475 μL). The pH was adjusted to 12 by the addition of NaOH (0.1 mol/L). The resulting solution was then titrated by HCl (0.1 mol/L, 10 μL each time) and monitored by pH meter.

The partition of dibenzyl-$_L$-glutamate (DBLG) in water/chloroform biphasic system

The model compound of oligomeric polypeptide, dibenzyl-$_L$-glutamate (DBLG), was first obtained by neutralizing its HCl salt. Briefly, DBLG hydrochloride was dissolved in DI water, and the aqueous pH was adjusted to 12 by NaOH. Chloroform was then added to extract DBLG.

Equal volume of DI water was added to the chloroform solution of DBLG. The mixture was vigorously vortexed to achieve equilibrium. An aliquot (20 μL) of aqueous and oil phases was taken out and dissolved in DMSO-d_6 (500 μL) with pre-dissolved ethanol as the internal standard. The ^{1}H NMR of each solution was characterized, and the amount of DBLG was quantified by comparing the signals of DBLG and ethanol.

DLS characterization of branched polypeptides

The DMF solution of PAMAM-PBLG (1 mg/mL) was filtered through nylon membrane (0.22 μm) twice to remove dust. Particle size and polydispersity were measured with a DLS detector. The theoretical size of PAMAM-PBLG was calculated by assuming α-helical conformation of PBLG, whose length was determined as (0.15 × degree of polymerization (DP)) nm, where the DP of PBLG was calculated based on the obtained MW by GPC. The size of PAMAM (generation 3.0) was obtained as 3.1 nm from literature report [4]. Therefore, the theoretical size of PAMAM-PBLG was estimated as (2 × 0.15 × DP + 3.1) nm.

Statistical analysis

Statistical analysis was performed using Student's *t*-test. The differences between two experimental groups were assessed to be significant at $*p < 0.05$ and very significant at $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$

Supporting Figures

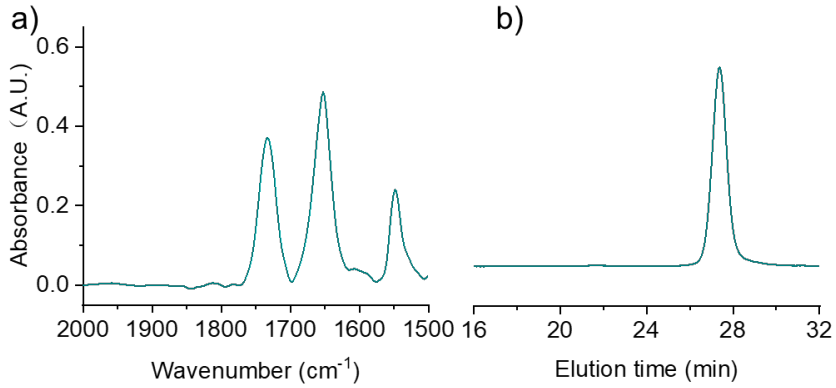


Fig. S1 Characterization of PEG-PBLG macroinitiators. (a) FTIR spectrum of PEG-PBLG. The Amide I peak at 1652 cm^{-1} and Amide II peak at 1548 cm^{-1} suggested an α-helical conformation of the PBLG segment. (b) GPC trace of PEG-PBLG. M_n = 10.5 kDa, $Đ$ = 1.05. The DP of PBLG segment was calculated to be 25.

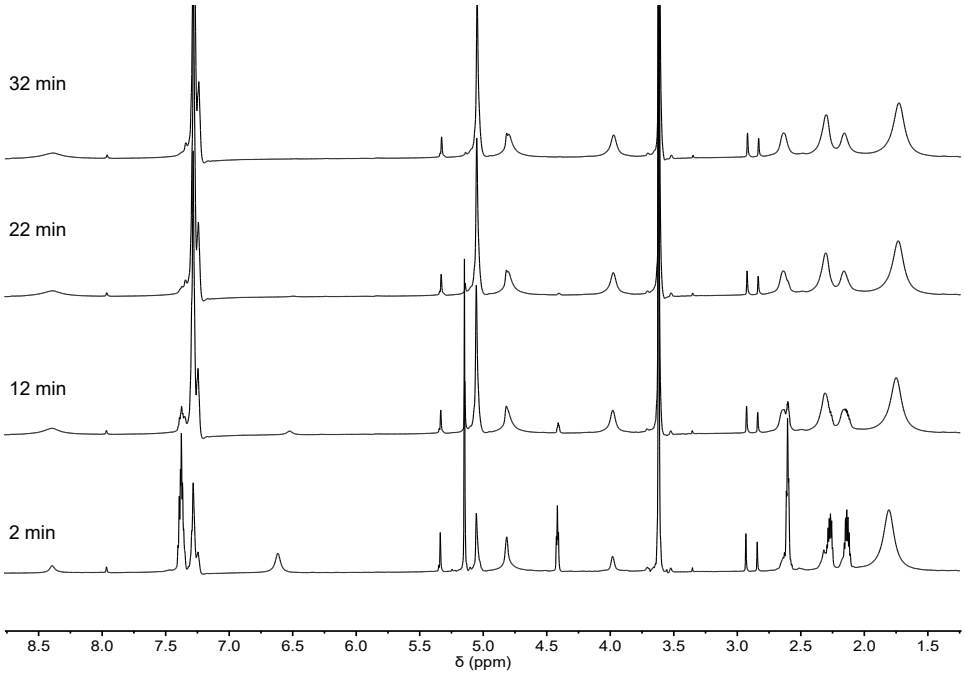


Fig. S2 Overlaid ^{1}H NMR spectra showing the polymerization kinetics of BLG-NCA in CD_2Cl_2 initiated by PEG-PBLG.

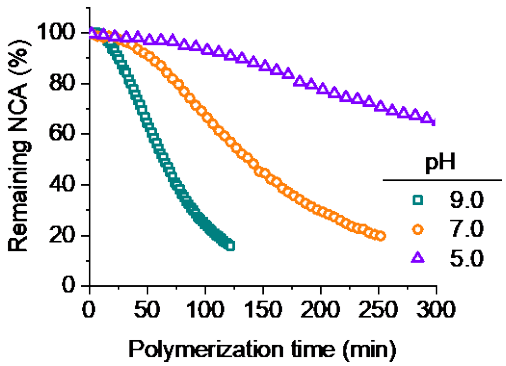


Fig. S3 Conversion of BLG-NCA in a water/CD_2Cl_2 biphasic system at various aqueous pH. $[NCA]_0$ = 0.1 mol/L, water:CD_2Cl_2 = 1:100 (w/w). The water-in-oil emulsion was stabilized with the addition of mPEG-NHAc emulsifier.

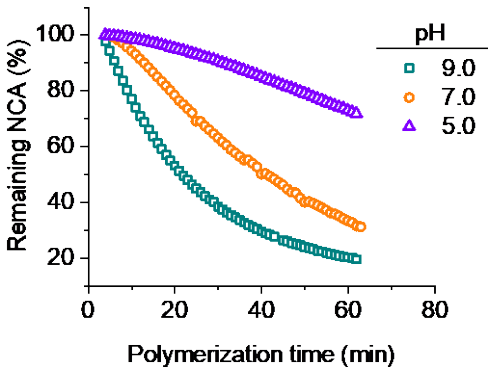


Fig. S4 Conversion of BLG-NCA in a water/$CDCl_3$ biphasic system at various aqueous pH. $[NCA]_0$ = 0.1 mol/L, water:$CDCl_3$ = 1:100 (w/w).

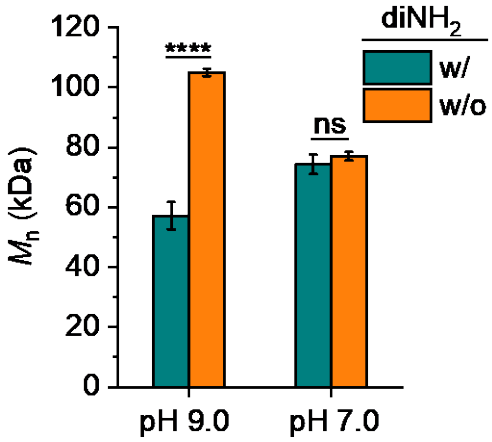


Fig. S5 GPC analysis of resulting polypeptides obtained from biphasic CCP in the presence and absence of C6-diNH$_2$ that was initially dissolved in the aqueous phase. Error bars represent the standard deviation from three independent polymerizations. ns = not significant.

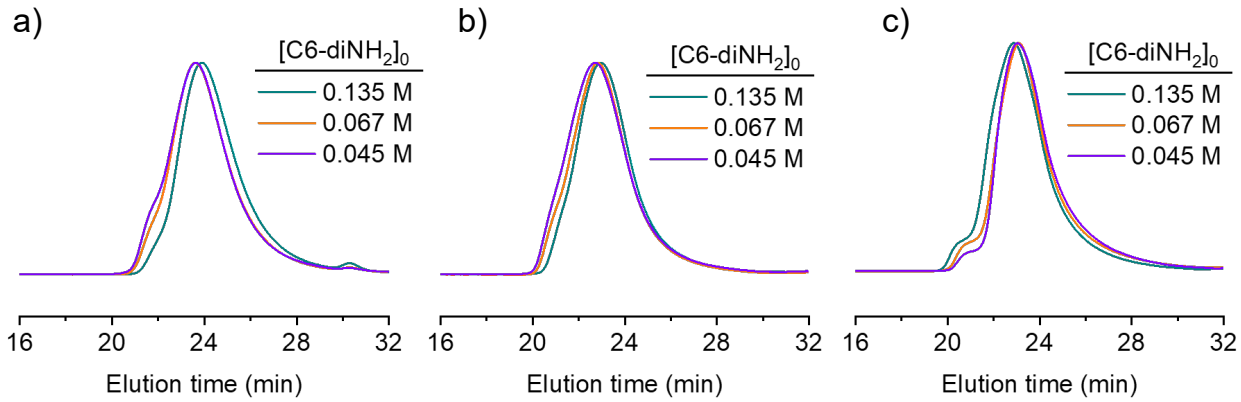


Fig. S6 GPC traces of resulting polypeptides obtained from biphasic CCP with different [C6-diNH$_2$]$_0$ at an aqueous pH of 9.0 (a), 7.0 (b), and 5.0 (c), with C6-diNH$_2$ initially dissolved in the aqueous phase.

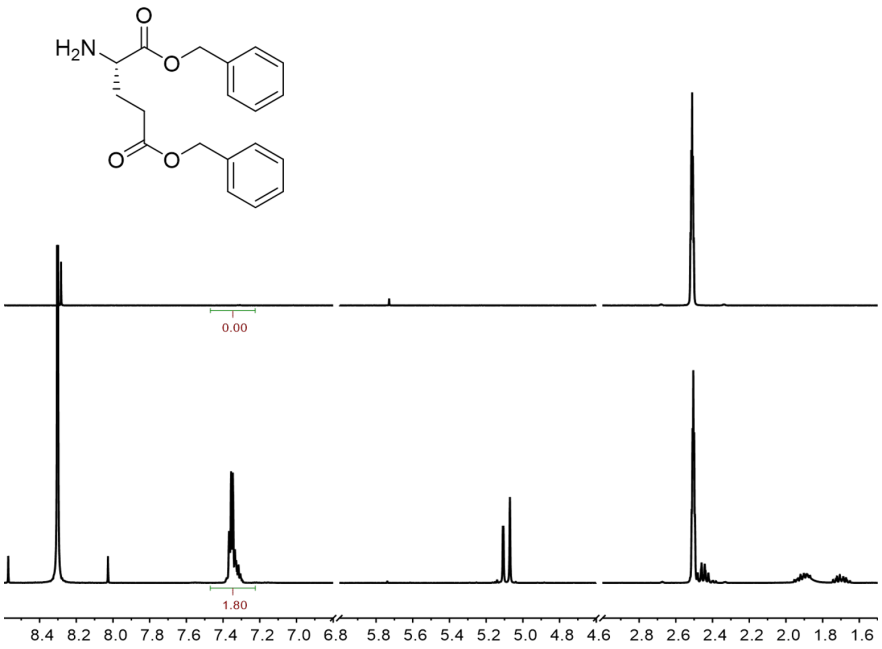
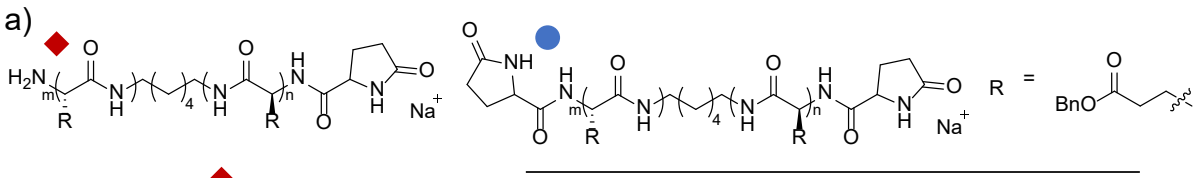
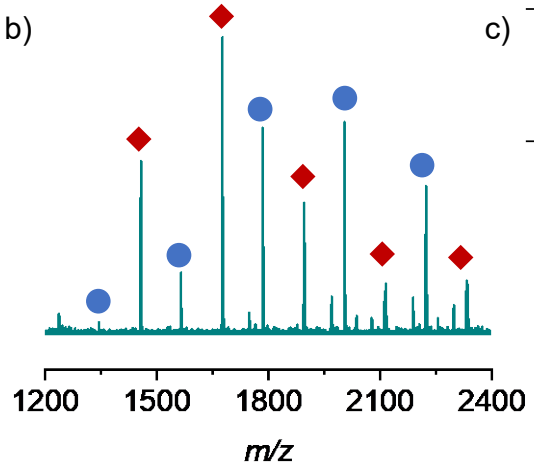


Fig. S7 ^{1}H NMR spectra (400 MHz, DMSO-d_6) measuring the partition of dibenzyl-L-glutamate (DBLG) in water (top) and chloroform (bottom). DBLG was used as a model compound of oligomeric PBLG. Ethanol was used as the internal standard to quantify the partition of DBLG.





c)

m+n	◆ species		● species	
	Calcd.	Found	Calcd.	Found
5	1345.6	1345.5	1456.6	1456.6
6	1564.7	1564.8	1675.7	1675.9
7	1783.8	1784.1	1894.8	1895.2
8	2002.9	2003.3	2113.9	2113.5
9	2222.0	2222.6	2333.0	2332.7

Fig. S8 MALDI-TOF MS characterization of polypeptides obtained from biphasic CCP with C6-diNH$_2$ originally dissolved in the chloroform phase. (a) Chemical structures of identified ionic polypeptide species on MALDI-TOF MS spectrum. (b) MALDI-TOF MS spectrum of polypeptides. (c) Comparison of representative *m*/*z* signals between calculated values from molecular formula and obtained values from MALDI-TOF spectrum.

The obtained *m*/*z* signals agreed well with the calculated values [250.15 + 219.09(*m* + *n*)] and [361.19 + 219.09(*m* + *n*)], corresponding to the polypeptides with the backbiting on one end or both ends ([M+Na]$^+$).

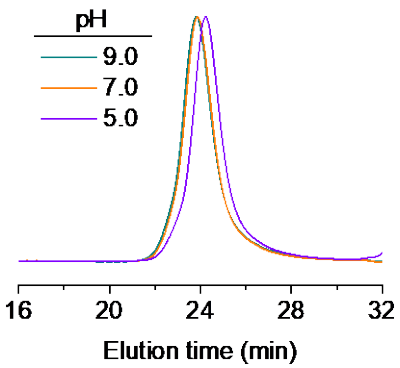


Fig. S9 GPC traces of resulting polypeptides obtained from PAMAM-initiated biphasic CCP at various aqueous pH, with PAMAM initially dissolved in the chloroform phase.

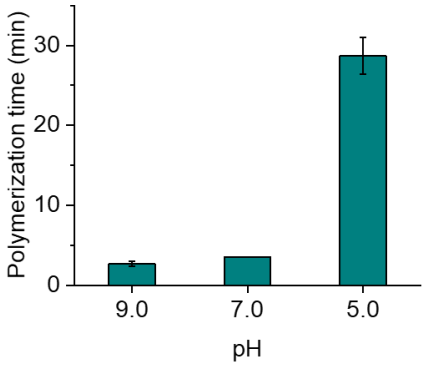


Fig. S10 Polymerization time reaching > 95% monomer conversion in a water/$CHCl_3$ biphasic system at various aqueous pH in the presence of PAMAM, as monitored by FTIR (n = 3). $[M]_0$ = 0.1 mol/L, $[M]_0/[I]_0$ = 50, water:$CHCl_3$ = 1:100 (w/w).

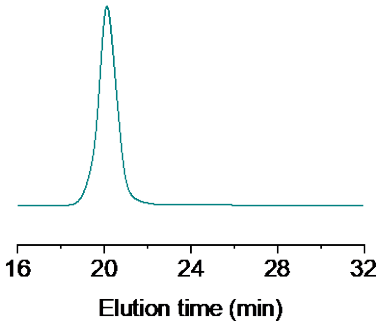
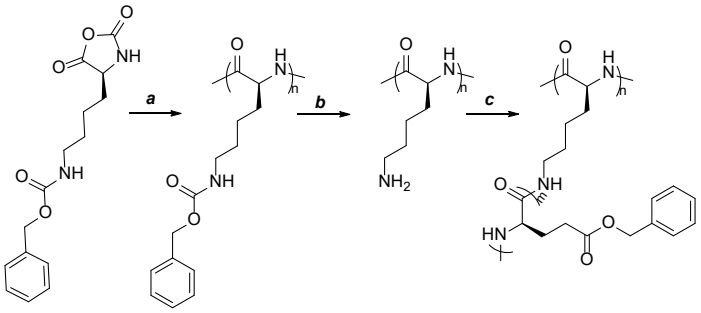


Fig. S11 GPC trace of PLL-PBLG brush polypeptides obtained from biphasic CCP, with PLL macroinitiators initially dissolved in the aqueous phase. M_n = 1190 kDa, M_n^* = 333.7 kDa, $Đ$ = 1.15. The larger obtained MW than the theoretical value was attributed to the partition and loss of partial PLL macroinitiators in the aqueous phase.

Supplementary Scheme



Scheme S1 Synthetic route to PLL-PBLG brush polypeptides. ***a***. *n*-hexylamine, DMF, overnight. ***b***. HBr (33% HOAc), TFA, 0 ºC to rt, 2 h. ***c***. BLG-NCA, $[M]_0$ = 0.1 mol/L, $[M]_0/[I]_0$ = 50, water:$CHCl_3$ = 1:100 (w/w), pH 7.0.

Supplementary Tables

Table S1 Biphasic CCP in the presence of various concentration of C6-diNH$_2$ that was initially dissolved in the aqueous phase.[a]

Entry	pH	[C6-diNH$_2$]$_0$	M_n/M_n^* (kDa) [b, c]	$Đ$[c]
1	9.0	0.067	63.0/44.0	1.18
2	9.0	0.045	65.0/67.1	1.20
3	7.0	0.067	102.8/44.0	1.15
4	7.0	0.045	106.6/65.9	1.18
5	5.0	0.067	83.2/44.0	1.19
6	5.0	0.045	75.4/65.9	1.20

[a] All polymerizations were conducted at room temperature in a water/chloroform biphasic system. [M]$_0$ = 0.1 mol/L, water:CHCl$_3$ = 1:100 (w/w). C6-diNH$_2$ was initially located at the aqueous phase. [b] Obtained MWs/Designed MWs*. [c] Determined by GPC; dn/dc = 0.098.

Table S2 Biphasic CCP in the presence of C6-diNH_2 that was initially dissolved in the chloroform phase.[a]

Entry	pH	M_n/M_n^* (kDa)[b, c]	$Đ$[c]
1	9.0	28.5/22.0	1.59
2	7.0	22.3/22.0	1.75
3	5.0	25.9/22.0	1.58
4[d]	-	27.6/22.0	1.58

[a] All polymerizations were conducted at room temperature in a water/chloroform biphasic system. $[M]_0$ = 0.1 mol/L, $[M]_0/[I]_0$ = 50, water:$CHCl_3$ = 1:100 (w/w). C6-diNH_2 was initially located at the chloroform phase. [b] Obtained MWs/Designed MWs*. [c] Determined by GPC; d*n*/d*c* = 0.098. [d] Polypeptide obtained from a solution polymerization in chloroform was used as a reference.

Table S3 Preparation of PAMAM-PBLG star polypeptides with biphasic CCP.[a]

Entry	$[M]_0/[I]_0$	t (min)[b]	M_n/M_n^* (kDa)[c,d]	$Đ$[d]
1	50	3.5	430/358	1.17
2	100	4.5	800/708	1.20
3	150	6	1130/1059	1.20

[a] All polymerizations were conducted at room temperature in a water/$CHCl_3$ biphasic system. $[M]_0$ = 0.1 mol/L, water: $CHCl_3$ = 1:100 (w/w), pH 7.0. PAMAM was initially located at the aqueous phase. [b] Polymerization time reaching 95% monomer conversion. [c] Obtained MWs/Designed MWs*. [d] Determined by GPC; dn/dc = 0.098.

Reference

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[4] L.J. Fox, R.M. Richardson, W.H. Briscoe, PAMAM dendrimer-cell membrane interactions, Adv. Colloid Interface Sci., 257 (2018) 1-18.