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Accelerated and controlled polymerization of *N*-carboxyanhydrides assisted by acids

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Abstract

It has been widely accepted that acidic species, such as HCl, inhibit the polymerization process of *N*-carboxyanhydrides (NCAs), which have to be removed to guarantee the successful synthesis of polypeptides. Herein, we showed that the impact of organic acids on NCA polymerization was dependent on their pKa values in dichloromethane. While stronger acids like trifluoroacetic acids completely blocked the chain propagation as expected, weaker acids such as acetic acids accelerated the polymerization rate instead. The addition of acids not only protonated the propagating amino groups but also activated NCA monomers, whose balance determined the accelerating or inhibitory effect. Additionally, the acid-assisted polymerization exhibited one-stage kinetics that differed from conventional cooperative covalent polymerizations, resulting in excellent control over molecular weights even with an accelerating rate. The pKa-dependence inspired us to turn the inhibitory acids into accelerating acids on demand, promoting the controlled polymerization from non-purified NCA monomers. This work highlights the possibility to change the conventional understanding of an activator /inhibitor by altering reaction conditions, which not only sheds light on the design of new accelerating strategy, but also offers a practical strategy to prepare polypeptide materials in an efficient and controlled manner.

Keywords

N-carboxyanhydride, polypeptide, organic acid, pKa value, organocatalysis

Introduction

Polypeptides from the ring-opening polymerization of amino acid *N*-carboxyanhydrides (NCAs) are regarded as the synthetic analogue of natural proteins,[1,2] which have shown promising applications in various biomedical fields including drug delivery, tissue engineering, and antimicrobial applications.[3-12] The advances in living polymerization of NCA over the last three decades enabled the preparation of polypeptides with predictable molecular weights (MWs) and low dispersity,[13-19] expanding the toolbox to prepare various polypeptide materials. In addition, the recent development of accelerated polymerization strategies not only shortened the polymerization time, but also outpaced various side reactions during NCA polymerization.[20-28] Polypeptides were prepared in a controlled manner even in the presence of aqueous phase,[29-31] which was impossible in conventional polymerization considering the water-induced NCA degradation.

Despite the exciting advances in NCA polymerization chemistry, the key limitation remains to delicately balance the basic/nucleophilic and acidic/electrophilic species during the handling of NCA monomers. While the former induced degradation of NCA monomers, the latter reacted with the propagating amino groups that blocked the polymerization process.[29,32,33] For instance, the widely used NCA synthetic strategy through Fuchs-Farthing method,[34,35] which involved the phosgenation of corresponding amino acids, generating HCl as one of the major impurities. HCl would inhibit the polymerization of NCAs, because its presence protonates amino groups and disfavors the formation of NCA anion, suppressing the polymerization with both normal amine mechanism (NAM) and activated monomer mechanism (AMM).[32,36] Therefore, HCl was commonly used to quench the polymerization for the analysis of polymerization intermediates.[37]

Experimental Methods

Polymerization setup and polypeptide characterization

Acetic acid (AcOH)-accelerated polymerization of NCA was carried out under ambient conditions. Typically, AcOH (2.17 μL, 0.038 mmol) was mixed with the DCM solution of BLG-NCA (10 mg, 0.038 mmol), into which the DCM solution of Hex-NH_2 (0.076 M, 5 μL, 0.38 μmol) was added to start the polymerization ($[M]_0$ = $[AcOH]_0$ = 0.1 M, $[M]_0/[I]_0$ = 100). After > 99% conversion of NCA as monitored by FTIR, the resulting polymers were purified by precipitation in hexane/ether (1:1, v/v) and dried under vacuum. The obtained polypeptides were dissolved in DMF containing 0.1 M LiBr, filtered through a 0.45 μm PTFE membrane (Thermo Fisher Scientific, Waltham, MA, USA), and analyzed by GPC. Polymerization in other solvents, with other monomers, initiators ($[M]_0/[I]_0$ = 50-200), and acids ($[acid]_0/[M]_0$ = 0.01-2) were conducted in a similar way. In order to check the MWs at different monomer conversions, the polymerization mixture was stopped at different time intervals through the addition of trifluoroacetic acid (TFA, 2.5 vol%). The polypeptides were then purified by precipitation, dried, and dissolved in DMF containing LiBr (0.1 mol/L) for GPC analysis. The secondary structure analysis of polypeptides was conducted in a similar way, but diluted by 100 times with DCM after quenching. Only the CD spectra at λ > 220 nm were measured owing to the absorbance of DCM at low-wavelength region.

Polymerization of non-purified BLG-NCA

To a pressure vessel with a heavy wall, γ-benzyl-$_L$-glutamate (10.0 g, 42.1 mmol), THF (150 mL), methyloxirane (13.0 mL, 169 mmol) were added sequentially under magnetic stirring. Triphosgene (6.3 g, 21.1 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 dried under vacuum without any additional purification procedures. The non-purified NCA could be stored at -20 ^oC for at least 1 year without significant degradation.

Polymerization of non-purified NCA with the acid-base equilibrium was carried out under ambient conditions. Typically, HCl (3 μL, 0.038 mmol) was mixed with the DCM solution of non-purified BLG-NCA (10 mg, 0.038 mmol), into which the DCM solution of Hex-NH_2 (0.076 M, 5 μL, 0.38 μmol) was added. After thorough mixing, NaOAc (3.12 mg, 0.038 mmol) is added into the mixture to convert the inhibitory HCl into catalytic AcOH ($[M]_0$ = $[HCl]_0$ = $[NaOAc]_0$ = 0.1 M, $[I]_0$ = 1 mM).

NMR titration

NMR titration experiments were conducted to probe the molecular interactions between the reactants during NCA polymerization, including BLG-NCA, Hex-NH_2, AcOH, and chain-end-mimicking α,γ-dibenzyl-$_L$-glutamate (DBLG). Taking the experiments to elucidate the NCA/acid interactions as an example, BLG-NCA (10.00 mg, 0.038 mmol) was dissolved in CD_2Cl_2 (380 μL), into which various amounts of AcOH were added (from 0.02 to 9.28 mg), so that the $[AcOH]_0/[NCA]_0$ ratio was varied from 0.01 to 1. The chemical shifts of α-H, side-chain benzyl protons, and ring N-H at different $[AcOH]_0/[NCA]_0$ ratios were recorded.

Other NMR titration experiments like Hex-NH_2/acid interactions and DBLG/acid were conducted in a similar manner. For Hex-NH_2/acid experiments, the fraction of Hex-NH_2 protonation was quantified by the chemical shift of α-H of Hex-NH_2, which was calculated according to the following equation:

$$\delta_m = \delta_b + (\delta_s - \delta_b) \cdot X_s$$

Where δ_b and δ_s are the standard chemical shifts of α-H in the amine (-NH_2) and ammonium (-NH_3^+) forms of Hex-NH_2, respectively, and δ_m is the chemical shift of α-H under certain conditions. The mole fraction of protonated amine, X_s, was then calculated from the equation.[38]

Results and Discussion

Accelerated polymerization assisted by acetic acid.

It has been reported that the ammonium salts served as initiators for the controlled polymerization of NCA or *N*-phenoxycarbonyl amino acids under certain conditions.[14,39,40] While the addition of excessive acids is generally believed to slow down or completely prohibit NCA polymerization,[32,36] organic acids are common catalysts for polyester synthesis, which activates the monomers through protonation or H-bonding interactions.[41-46] Considering the structural similarity of NCA with lactones, we reasoned that the addition of acids would also activate NCA. Nevertheless, HCl generated during the synthesis of NCA deactivates the nucleophilic initiators or propagating polypeptide chains during NCA polymerization, because the basic nature of the propagating chain-ends would react with the relatively strong acids (i.e., HCl) that leads to chain inhibition even with potentially activated monomers. On the other hand, it has been reported that the amino group still has sufficient activity towards *N*-hydroxy succinimide (NHS) ester for nucleophilic substitution at pH 4-5, where amine is only partially protonated with a considerable fraction staying in its nucleophilic state.[47] Therefore, a weak organic acid may still serve as an accelerating agent for NCA polymerization, which activates the monomer while still keeps sufficiently high amine chain-end reactivity.

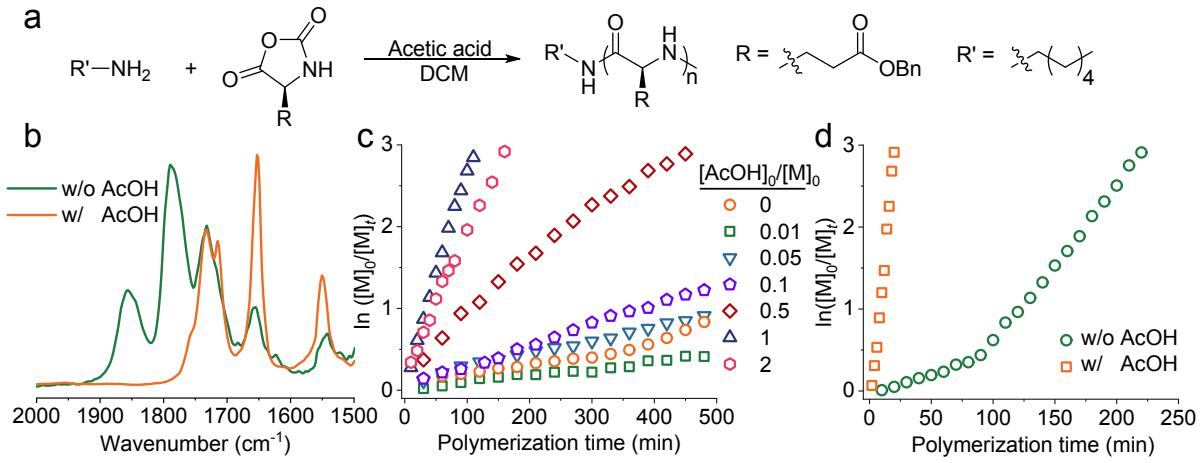

Figure 2. AcOH-accelerated polymerization of NCAs. (a) Synthetic route to polypeptides through AcOH-accelerated polymerization of BLG-NCA. (b) Overlaid FTIR spectra showing the polymerization of BLG-NCA initiated by Hex-NH_2 after 120 min in the absence or presence of AcOH in DCM. $[M]_0/[I]_0/[AcOH]_0$ = 100/1/100, $[M]_0$ = 0.1 M. (c) Semilogarithmic kinetic plot of polymerization of BLG-NCA in DCM initiated by Hex-NH_2 at various $[AcOH]_0$. $[M]_0/[I]_0$ = 100, $[M]_0$ = 0.1 M. (d) Semilogarithmic kinetic plot of polymerization of BLG-NCA in DCM initiated by Hex-NH_2 at low $[M]_0/[I]_0$ in the absence or presence of AcOH. $[M]_0/[I]_0/[AcOH]_0$ = 50/1/100, $[M]_0$ = 0.1 M.

In order to verify our hypothesis, AcOH with a high pKa value (~ 4.76)[48] was first added into the DCM solution of γ-benzyl-$_L$-glutamate NCA (BLG-NCA) and *n*-hexylamine (Hex-NH_2) ($[M]_0$ = 0.1 M, $[M]_0/[I]_0/[AcOH]_0$ = 100:1:100) (Figure 2a). Fourier-transform infrared (FTIR) characterization revealed the complete disappearance of NCA anhydride peaks at 1857 cm^{-1} and 1790 cm^{-1} after 120 min in the presence of AcOH (Figure 2b). In contrast, the NCA conversion was only 20% in the absence of AcOH after 120 min (Figure 2b), suggesting the accelerating role AcOH played during NCA polymerization. Additionally, NCA was stable in the presence of AcOH without the addition of Hex-NH_2 initiator (Figure S1), ruling out the possibility that AcOH served as accelerated initiators.

The accelerating effect of AcOH was dependent on the amount of the acid. The polymerization kinetics of BLG-NCA was monitored in situ by FTIR, which showed a low rate with 47% conversion after 7 h ($[M]_0$ = 0.1 M, $[M]_0/[I]_0$ = 100:1) (Figure 2c). The addition of a small amount of AcOH, with an equimolar concentration to the initiator, slightly slowed down the polymerization that only 30% of NCA was consumed after 7 h. The increase in the $[AcOH]_0/[M]_0$ ratio accelerated the NCA polymerization, with the NCA conversion of 56%, 67%, and 94% after 7-h polymerization at $[AcOH]_0/[M]_0$ = 0.05, 0.1, and 0.5, respectively. The polymerization reached the highest rate at $[AcOH]_0/[M]_0$ = 1, with an equimolar concentration of AcOH and monomer, where the polymerization reached > 95% conversion within 2 h (Figure 2c and Figure S2). A further increase in the $[AcOH]_0/[M]_0$ ratio to 2, however, slightly slowed down the polymerization kinetics. Additionally, with the same

batch of BLG-NCA, AcOH exhibited similar accelerating behavior with crown ether (CE), 18-crown 6-ether, which led to faster kinetics in comparison with conventional catalytic polymerization systems in DCM, including tetrabutylammonium acetate (TBAA) initiating system and *N,N'*-bis[3,5-bis(trifluoromethyl)phenyl]thiourea (TU-S) catalytic system (Figure S3). Notably, the fastest kinetics was achieved at $[AcOH]_0/[M]_0$ = 0.1 in *N*-thiocarboxyanhydrides (NTAs)/ amino acid *N*-substituted *N*-carboxyanhydrides (NNCAs) polymerization in nonpolar solvents, and achieved at $[AcOH]_0/[M]_0$ = 2 in *N*-substituted glycine *N*-thiocarboxyanhydrides (NNTAs) polymerization in polar solvent.[49-54] The latter required much more dosage of organic acids owing their consumption by polar solvent. The difference in kinetics among various monomers indicated different polymerization mechanisms.

The optimal ratio of AcOH was also dependent on the $[M]_0/[I]_0$, since similar kinetics was observed at $[AcOH]_0/[M]_0$ = 1 and 2 for a 50-mer polypeptide synthesis (Figure S2). The kinetic studies of AcOH mediated polymerization also presented a linear correlation with a slope of 0.05, indicating a first-order dependence of the polymerization rate dependence on initiator concentration, consistent with features of living polymerization (Figure S4). In addition to $[AcOH]_0$, $[M]_0$ also played an important role in the polymerization kinetics, which was consistent with the previous studies of polymerization in DCM.[55] The increase in $[M]_0$ significantly accelerated the polymerization, as the polymerization rapidly finished within 25 min in the presence of AcOH at $[M]_0$ = 0.2, 0.3, and 0.4 M (Figure S5).

In contrast to the previous reports on the cooperative covalent polymerization (CCP) in DCM,[22,55-58] the kinetic plot of AcOH-accelerated polymerization did not show an obvious two-stage kinetics. In order to further study the detailed kinetic profiles, the polymerization of BLG-NCA was conducted at low $[M]_0/[I]_0$ to clear reveal the early-stage kinetics. As shown in Figure 2d, the polymerization in the absence of AcOH exhibited a clear two-stage kinetics, agreeing well with previous studies.[22] The two-stage kinetics of CCP originated from the change in secondary structure, where the folding of polypeptides into α-helices accelerated the polymerization that outpaced the random-coiled chains.[22] In sharp contrast, the polymerization exhibited a one-stage kinetics in

the presence of AcOH (Figure 2d), which was further confirmed by the in situ NMR kinetic plot (Figure S6). Interestingly, CD characterization suggested the formation of random-coiled structure during AcOH-accelerated polymerization (Figure S7), excluding the possibility that there is no conformational change in the presence of AcOH. Therefore, the addition of AcOH altered the polymerization profile by accelerating both the random-coiled and α-helical propagating chains, resulting in a relatively stable rate constant throughout the polymerization process.

Improved control over molecular weights originated from the one-stage kinetics.

One of the main limitations of CCP is the bimodal distribution of resulting polypeptides, which originates from the two-stage kinetics with a faster secondary stage.[55,58] The propagating chains entering the second stage outgrow those staying in the first stage, resulting in an obvious shoulder peak at the low-MW side on the gel permeation chromatography (GPC) trace, especially in some fast CCP systems at low $[M]_0/[I]_0$.[22,59] The design of even faster CCP systems is thus disfavored due to the concerns in MW control. Previous literatures had to rely on the use of α-helical macroinitiators or cosolvents to skip the first stage or ameliorate the two-stage kinetic profile, respectively.[29,59,60]

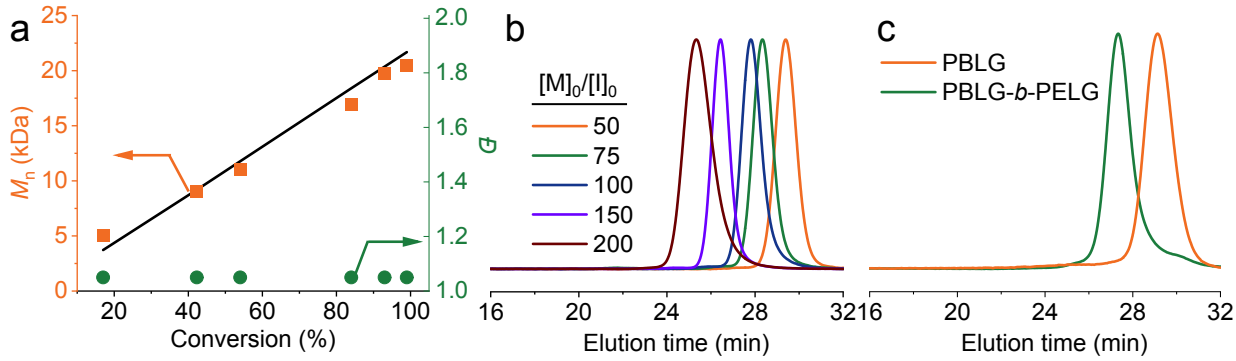

Figure 3. Molecular weight control of AcOH-accelerated polymerization. (a) The obtained MWs and dispersity at various monomer conversion during AcOH-accelerated polymerization of BLG-NCA initiated by Hex-NH_2 in DCM. $[M]_0/[I]_0/[AcOH]_0$ = 100/1/100, $[M]_0$ = 0.1 M. (b) Normalized GPC-LS traces of the obtained PBLG from

AcOH-accelerated polymerization at different $[M]_0/[I]_0$ ratios. (c) Normalized GPC-LS traces showing the synthesis of diblock copolypeptides PBLG-*b*-PELG in the presence of AcOH. $[M]_0/[I]_0/[AcOH]_0$ = 50/1/100, $[M]_0$ = 0.1 M.

The AcOH-accelerated polymerization with an accelerated, one-stage kinetics offers a promising strategy to solve the rate/MW-control dilemma, as all propagating chains grew simultaneously at a fast rate in the presence of AcOH. The plot of MWs against monomer conversion revealed a linear relationship, suggesting a living polymerization process (Figure 3a). Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry indicated negligible degradation of propagating amino groups (Figure S8), which was attributed to the accelerated rate. Notably, though carboxylate-initiated ring-opening polymerization (ROP) of NCAs has been previously reported,[28] no carboxylate end groups was observed in our study, suggesting that the polypeptides were exclusively initiated by the Hex-NH_2 initiator even in the presence of a high concentration of AcOH. The uniform initiation was attributed to the higher nucleophilicity of amino groups compared to the carboxylate groups. The obtained MWs of resulting polypeptides agreed well with the theoretical values when the feeding $[M]_0/[I]_0 \leq 200$, with a narrow dispersity observed for all polymerizations ($Đ = M_w/M_n < 1.10$) (Figure 3b and Table 1). Additionally, commercial available TBAA and CE results in a significant deviation from the targeted MWs compared to AcOH (Figure S9 and Table S1). At even higher $[M]_0/[I]_0$, the polymerization slowed down significantly that the obtained MWs were lower than the theoretical values. An increase in $[M]_0$ was therefore essential to guarantee the fast kinetics to obtain a poly(γ-benzyl-$_L$-glutamate) (PBLG) 400-mer (Figure S10). It has to be noted that the molecular weight distribution (MWD) of obtained polypeptides with $[M]_0/[I]_0 < 50$ was significantly improved in the presence of AcOH compared to that in the absence of AcOH (Figure S11 and Table S2), substantiating the improved MW control of AcOH-accelerated polymerization. The accelerated, controlled polymerization in the presence of AcOH was further extended to other monomers and initiators. Well-defined polypeptides were obtained from the polymerization of N^{ε}-carboxybenzyl-$_L$-lysine NCA (ZLL-NCA) and γ-(4-propargyloxy)benzyl-$_L$-glutamate NCA (POB-NCA), the

latter with a functional alkyne side chain for further modifications (Figure S12 and Table S3). Additionally, the livingness of AcOH-accelerated chains allowed for efficient chain extension to prepare block copolypeptides. GPC characterization revealed a clear peak shift with a high blocking efficiency (Figure 3c and Table S4). Other primary amines, including benzylamine, propagylamine, and methoxy poly(ethylene glycol) (PEG) amine, were also used as the initiator for the AcOH-accelerated polymerization (Figure S12 and Table S3). The successful incorporation of the functional group at the C terminus allowed for further polypeptide functionalization (Figure S13).

Table 1. Characterization of the resulting PBLG from the polymerization of BLG-NCA at various $[M]_0/[I]_0$ in the presence of AcOH.[a]

Entry	$[M]_0/[I]_0/[AcOH]_0$	t (min)[b]	$M_{n,GPC}$ (kDa)[c]	$M_{n,theo.}$ (kDa)	$Đ$ [c]
1	100/1/0	1440	19.9	21.9	1.12
2	50/1/100	60	11.1	10.9	1.05
3	75/1/100	70	16.5	16.4	1.05
4	100/1/100	120	20.5	21.9	1.05
5	150/1/100	300	32.4	32.8	1.05
6	200/1/100	480	41.2	43.8	1.05

[a]All polymerizations were conducted at room temperature in DCM with BLG-NCA as the monomer and Hex-NH_2 as the initiator. $[M]_0$ = 0.1 M. [b]Polymerization time reaching 95% monomer conversion. [c]Determined by GPC; dn/dc = 0.104.

pKa-Dependent accelerating or inhibitory effect of organic acids.

The accelerated polymerization of NCA in the presence of AcOH, in contrast to our previous understanding on the inhibitory effect of acid, inspired us to further explore the impact of more organic acids on the polymerization profile (Figure 4a). The addition of trifluoroacetic acid (TFA), an acid with a much lower pKa value (pKa ~ 0.52) than AcOH,[48] completely inhibited the polymerization with negligible monomer conversion

after 9 h (Figure 4b), suggesting that the pKa value of the acid played an important role in determining the polymerization behaviors. With the addition of bromoacetic acid (BrA, pKa ~ 2.90), formic acid (FA, pKa ~ 3.77) and benzoic acid (BnA, pKa ~ 4.20),[48] low conversion of NCA monomers was observed after 9 h, with the monomer conversion increased monotonously with weaker acidity, reaching ~ 33%, 56%, and 81%, respectively. Finally, the selection of even weaker acids exhibited negligible difference on the accelerating effect, since the addition of propanoic acid (PrA, pKa ~ 4.87) and trimethylacetic acid (TMA, pKa ~ 5.03) accelerated the polymerization in a similar way with that of AcOH,[48] but with a slightly slower rate.

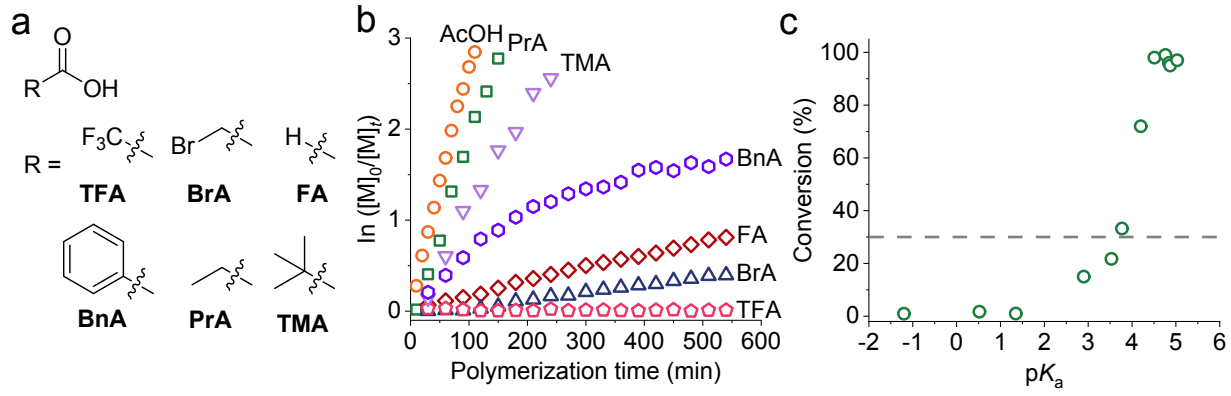

Figure 4. pKa-Dependent accelerating/inhibitory effect of organic acids. (a) Chemical structures of various organic acids with different pKa values. (b) Semilogarithmic kinetic plot of polymerization of BLG-NCA in DCM initiated by Hex-NH_2 in the presence of various organic acids. $[M]_0/[I]_0/[acid]_0$ = 100/1/100, $[M]_0$ = 0.1 M. (c) The plot of the 4-h NCA conversion against the pKa value of added organic acids. The grey, dashed line indicates the 4-h NCA conversion in the absence of any organic acids. $[M]_0/[I]_0/[acid]_0$ = 100/1/100, $[M]_0$ = 0.1 M.

By plotting the monomer conversion after 4-h polymerization against the pKa of various acids, it was clear that the polymerization kinetics exhibited a strong dependence on the acidity (Figure 4c and Table S5). While stronger acids (pKa < 1.5) completely inhibit the polymerization, acids with higher pKa values showed accelerating effect when pKa > 4. The conversion of NCA was > 95% within 4 h in the presence of acids with pKa > 4.5. It has to be noted that the polymerization kinetics in the presence of FA resembled that in the

absence of any organic acids. Therefore, FA was regarded as a model organic acid to differentiate the organic acids with accelerating or inhibitory effect depending on their acidity. GPC analysis revealed well-defined polypeptides with low dispersity ($Đ = M_w/M_n < 1.2$) for all acid-accelerated polymerizations (Table 2).

Table 2. Characterization of resulting PBLG initiated from the polymerization of BLG-NCA accelerated by various organic acid.[a]

Entry	Organic acid[b]	t (min)[c]	$M_{n,GPC}$ (kDa)[d]	$Đ$ [d]
1	PrA	160	22.6	1.05
2	TMA	240	21.0	1.05
3	IA	240	22.2	1.05
4	CA	240	24.7	1.06
5	BnA	720	20.1	1.14

[a]All polymerizations were conducted at room temperature in DCM with BLG-NCA as monomer and Hex-NH_2 as the initiator. The theoretical MW was 21.9 kDa. $[M]_0/[I]_0/[acid]_0 = 100/1/100$, $[M]_0 = 0.1$ M. [b]PrA = propanoic acid, TMA = trimethylacetic acid, IA = isobutyric acid, CA = cyclohexylacetic acid. BnA = benzoic acid. [c]Polymerization time reaching 95% monomer conversion. [d]Determined by GPC; dn/dc = 0.104.

Mechanism Studies.

The pKa-dependent effect of acids on NCA polymerization behaviors encouraged us to elucidate the underlying mechanism. We first checked the solvent effect of AcOH-accelerated polymerization. The accelerated and controlled polymerization of NCA was only observed in solvents with low polarity and weak hydrogen bonding ability, such as DCM and chloroform (Figure 5a and Figure S14). While the polymerization in polar *N*,*N*-dimethylformamide (DMF) in the presence of AcOH exhibited a relatively fast rate (Figure S14), the resulting polypeptides exhibited a bimodal MWD (Figure 5a and Table S6), likely due to the presence of two polymerization process initiated by both the amine and ammonium form of Hex-NH_2. The polymerization in

tetrahydrofuran (THF), on the other hand, proceeded in a much slower rate and generated polypeptides with a broad MWD (Figure 5a, Figure S14 and Table S6). Since there were significant solvations of NCA monomers and propagating polypeptide chains in DMF and THF,[55] the solvent dependence suggested that the molecular interactions played important roles during AcOH-accelerated effect.

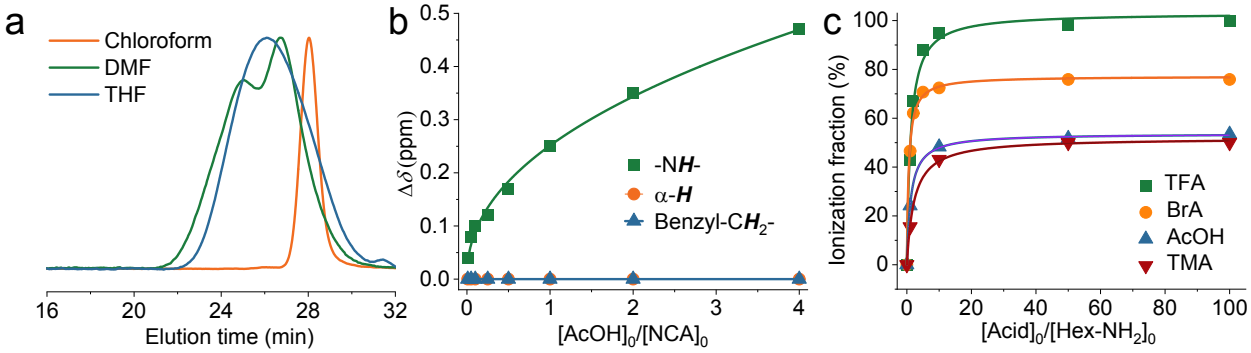

Figure 5. Elucidation of accelerating/inhibitory mechanisms. (a) Normalized GPC-LS traces of the obtained PBLG in various solvents initiated by Hex-NH_2 in the presence AcOH. $[M]_0/[I]_0/[AcOH]_0$ = 100/1/100, $[M]_0$ = 0.1 M. (b) The change in chemical shift of various protons in NCA at various $[AcOH]_0/[NCA]_0$ ratios. $[NCA]_0$ = 0.1 M. (c) The ionization fraction of Hex-NH_2 in the presence of various organic acids at different $[Acid]_0/[Hex\text{-}NH_2]_0$.

The rate-limiting step of NCA polymerization was the nucleophilic attack of propagating amines on the NCA anhydride.[44] In order to simplify the system, the kinetics of ring-opening reaction was monitored in the presence and absence of AcOH, with the mixing of BLG-NCA and chain-end-mimicking α,γ-dibenzyl-$_L$-glutamate (DBLG) at a ratio of 1:10 (Figure S15).[59] The presence of AcOH significantly accelerated the ring-opening reaction, with the NCA completely consumed within 6 min. In sharp contrast, the conversion of NCA was only 15% after 40 min in the absence of AcOH, suggesting that the accelerating effect mainly involved the local interactions at the N terminus, with negligible contribution from the overall polymeric structure. Moreover, the accelerating effect was also verified through a model reaction between DBLG and maleic anhydride (Figure

S16), indicating that the pKa-dependence was a general phenomenon regardless of the structure of the electrophile.

Learning from the organo-accelerating ROP to prepare polyesters and polycarbonates,[41,43,45,46] we reasoned that the accelerating effect of AcOH originated from the carbonyl activation of NCA monomers (Figure 1). The H-bonding interactions between NCA and AcOH lowered the energy of the lowest unoccupied molecular orbital (LUMO) of anhydride groups,[61] leading to rate enhancement. On the other hand, the pKa-dependent accelerating/inhibitory effect of acids likely resulted from the protonation of propagating amino groups (Figure 1). While weaker acids such as AcOH reversibly protonated the amino groups at the chain-end, stronger acids like TFA blocked the nucleophilic reaction by completely protonating the terminal amines. To verify our hypothesis, ^{1}H NMR and ^{13}C NMR titration experiment was performed to study the interactions between NCA and AcOH.[45,59,62] The addition of AcOH into the solution of BLG-NCA induced obvious downfield shift of the proton around 6.5 ppm in ^{1}H NMR and C2 carbon around 152 ppm in ^{13}C NMR (Figure 5b, Figure S17 and Figure S18), corresponding to the deshielding of the ring N-H proton.[63] The shift of α-H and side-chain benzyl protons, however, was negligible compared to that of ring N-H, suggesting that the NCA interacted with AcOH through H-bonding interactions at the ring amide structure. The slight downfield shift of C5 carbon of NCA around 172 ppm showed that the activation of C5 carbonyl group via a through-bond conductivity effect (Figure S18).[64,65] The α-carbon was also downfield-shifted due to through-bond conductivity effect (Figure S18).[64,65] The interactions activated the carbonyl group of NCA ring, rendering it more susceptible to nucleophilic attack, which facilitated the ring-opening reaction. Moreover, TFA induced an even larger shift of ring N-H (Figure S17), indicating an even stronger affinity with NCA.[66] Nevertheless, the protonation of propagating primary amino groups, as calculated from the chemical shift of methylene groups adjacent to amino groups in Hex-NH_2 (Figure S19, see Supporting Information for details),[38,67] was > 90% in the presence of 10 equiv. of TFA (Figure 5c). Therefore, even though TFA exhibited a stronger activation of NCA monomer than AcOH, the complete blocking of nucleophilic amines led to the inhibitory effect. In comparison, organic acids with weaker acidity showed

partial protonation of Hex-NH_2 even at $[acid]_0/[Hex\text{-}NH_2]_0$ = 100 (Figure 5c). For instance, 47% Hex-NH_2 stayed in the nucleophilic form in the presence of 100 equiv. of AcOH, which allowed for the accelerated polymerization with activated monomer. Additionally, the multiplicity of the methylene groups also indicated complete protonation of Hex-NH_2 in the presence of TFA (Figure S19). The simultaneous growth of all polypeptide chains, as evidenced by the low dispersity of resulting polypeptides, presumably originated from the fast, reversible exchange of protons among all propagating chains.[14] Moreover, a density functional theory (DFT) calculation revealed a significantly lower Gibbs free energy of the transition state of the ring-opening reaction in the presence of AcOH (10.1 kcal/mol) compared to that in the absence of AcOH (16.3 kcal/mol) (Figure S20), confirming the accelerating role of AcOH. In addition, AcOH also promoted the decarboxylation of carbamate species to accelerate the polymerization, as evidence by lower Gibbs free energy of the transition state (TS2: 34.8 kcal/mol *VS* TS3-AcOH: 12.4 kcal/mol) (Figure S21).

The one-stage kinetics of AcOH-accelerated polymerization was attributed to the H-bonding interactions between acids and polypeptide chains, which minimized the binding differences of NCA with α-helical and random-coiled propagating chains.[58,60] NMR titration studies suggested various H-bonding interactions between AcOH and DBLG at the backbone and side-chain carbonyl groups (Figure S22), substantiating the use of an equimolar ratio of AcOH to NCA to reach the fastest kinetics (Figure 2c). Without sufficient AcOH in the system, the competing H-bonding interactions with AcOH between polypeptide side chains and NCA weakened the activation of monomers that slowed down the polymerization process.

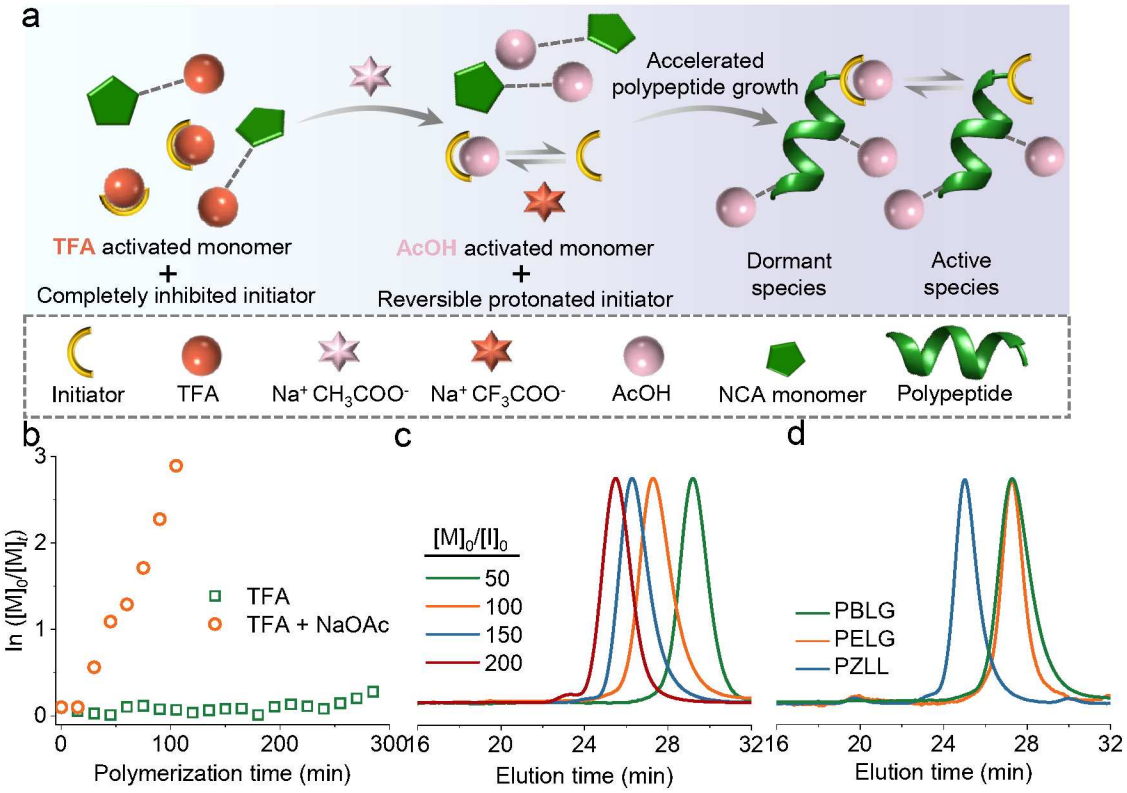

Figure 6. Polymerization of non-purified NCA. (a) Scheme illustration showing the "turn on" process by converting the inhibitory acids into accelerating ones. (b) Semilogarithmic kinetic plot of polymerization of BLG-NCA initiated by Hex-NH_2 in DCM in the presence of TFA and TFA + NaOAc. $[M]_0/[I]_0/[TFA]_0$ = 100/1/100, $[M]_0$ = $[NaOAc]_0$ = 0.1 M. (c) Normalized GPC-LS traces of the resulting polypeptides obtained from the polymerization of non-purified BLG-NCA at different $[M]_0/[I]_0$ ratios in the presence HCl and NaOAc. $[M]_0/[I]_0/[HCl]_0$ = 100/1/100, $[M]_0$ = $[NaOAc]_0$ = 0.1 M. (d) Normalized GPC-LS traces of the resulting polypeptides obtained in the presence NaOAc with non-purified BLG-NCA, ZLL-NCA and ELG-NCA as monomers. $[M]_0/[I]_0/[HCl]_0$ = 100/1/100, $[M]_0$ = $[NaOAc]_0$ = 0.1 M.

Polymerization of non-purified NCA with the acid-base equilibrium.

The pKa-dependent impact of organic acids on NCA polymerization allowed us to "turn on" NCA polymerization on demand, because the inhibitory, stronger acid spontaneously turned into accelerating, weaker acid upon

the addition of the conjugate base of the weak acid (Figure 6a). The presence of strong organic acids like TFA enhanced the NCA stability against moisture, as the amino acid from the hydrolysis of NCA was unable to further polymerize other monomers. Indeed, the addition of water into the NCA/TFA mixture resulted in negligible degradation of NCA monomers (Figure S23). Therefore, it is possible to store NCA monomers pre-mixed with initiators with the protection of TFA which was then converted to accelerating, weaker acid to initiate the polymerization.

In order to check our hypothesis, TFA was pre-mixed with BLG-NCA at an equimolar ratio. No polymerization was observed 24 h after the addition of Hex-NH_2, substantiating the inhibitory effect of TFA. The addition of sodium acetate (NaOAc) converted the inhibitory TFA into accelerating AcOH, where full NCA conversion was observed after 120 min (Figure 6b). The resulting polypeptides exhibited predictable MWs and low dispersity (Figure S24), demonstrating the robustness of AcOH-accelerated polymerization.

The acid-base equilibrium strategy was also used to convert HCl, one of the major impurities generated during NCA synthesis,[29,32,68] into accelerating AcOH during the direct polymerization of non-purified NCA. Previous reports had to rely on tedious and time-consuming anhydrous purification methods to remove impurities,[32,36] which are challenging to non-experts. Notably, nonpurified BLG-NCAs were obtained with a significantly higher isolation yield (> 95%) compared to 60~80% yield achieved after purification. Additionally, this method allowed for a much shorter processing time (~2 hours), as opposed to the days required for NCA purification. Thus, a strategy which enables controlled polymerization of nonpurified NCA monomers would be of interest and impact. Chlorine analysis confirmed the exitence of trace amount of impurities, in which the chlorine content of non-purified NCA was ~100 times that of purified NCA (Table S7). In the case of nonpurified BLG-NCAs, the presence of impurities such as HCl hindered the polymerization process when using a conventional polypeptide synthetic method. Upon mixing nonpurified BLG-NCAs with Hex-NH_2 in DCM, the NCA conversion was found to be less than 1% even after 12 h (Figure S25). While it was possible to remove HCl in situ through biphasic segregation, a sufficiently fast polymerization was necessary to outpace water-induced side reactions.[29] The

direct addition of NaOAc into the DCM solution of non-purified NCA successfully eliminated the inhibitory effect of HCl, but yielded ill-defined polypeptides (Figure S24), which was attributed to the initiation from the excessive acetate anions. Therefore, concentrated HCl was first added into the solution of non-purified NCA, which was then treated with NaOAc. PBLG end-capped with *n*-hexyl groups from non-purified NCAs was obtained, resembling its counterpart from purified NCAs (Figure S26). Furthermore, polypeptides with various side chains and MWs were efficiently obtained with low dispersity (Figure 6c-d, Figure S27 and Table S8), substantiating the use of acid-base equilibrium to directly polymerize non-purified NCA in a fast and controlled manner.

CONCLUSION

In summary, we reported that the addition of organic acids, previously regarded as detrimental to the NCA polymerization process, enabled the accelerated and controlled preparation of polypeptide materials. The acidity of the acids was critical to avoid complete blocking of propagating chains while activating NCA monomers. The use of solvents with low polarity and weak H-bonding ability, like DCM and chloroform, promoted the molecular interactions and played an important role in uncovering the new role of acids in NCA polymerization. The current study highlights the possibility to alter the previous understanding on NCA polymerization profile through the change in polymerization conditions, inspiring the design of new accelerating strategy.

Supporting Information

Supporting Information is available and includes materials, instrumentations, detailed experimental methods, NMR, FTIR, GPC, CD, MALDI-TOF data and calculated Gibbs free-energy profile (Figure S1-S27 and Table S1-S8).

Author Contributions

#These authors contributed equally.

Conflict of Interest

There is no conflict of interest to report.

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