Terpolymerization Kinetics of Amino Acid N-Carboxy Anhydrides

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Received 11 November 2013; accepted 14 January 2014; published online 12 February 2014
DOI: 10.1002/pola.27109

ABSTRACT: Based on their versatility with respect to amino acid type and sequence, polypeptides have become attractive for a number of biological applications such as drug delivery, biomineralization, and drugs. N-carboxy anhydride (NCA) polymerization is a convenient way to rapidly prepare high-molecular weight polypeptides with good control over molecular weight and polydispersity. However, the kinetics of the incorporation of NCA monomers into copolypeptides during random copolymerization are poorly understood. Here, kinetic data is presented that allows insight into the NCA polymerization of a terpolymer composed of three commercially relevant amino acids, namely, glutamic acid, lysine, and tyrosine. Furthermore, kinetic data and copolymerization parameters from the copolymerization of binary mixtures of these three amino acid NCAs is used to make predictions of the terpolymer composition. This study provides access to the information necessary to prepare functional copolypeptides with better-defined sequence architecture that will be essential for the future development of polypeptide-based materials. © 2014 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2014**, *52*, 1228–1236

KEYWORDS: copolymerization; glutamic acid; lysine; polymerization kinetics; polypeptides; tyrosine

INTRODUCTION In biology, polypeptides are essential components of cellular processes. Both the chemical variety of amino acids and the synergistic functionality that arises from specific amino acid sequences make polypeptides versatile molecules that perform a multitude of functions such as enzymatic catalysis, recognition events, and regulating processes.[1] The potential of peptides in materials science has been recognized[1–3] and over the last decades increasing efforts have been placed into designing functional peptide-based materials such as drug delivery vehicles,[4–10] peptide surfaces,[11–14] and self-assembling peptide-based gels.[15–21] As a result, preparation methods of peptides become more advanced. The preparation of polypeptides through N-carboxy anhydrides (NCA) polymerizations has been employed since the 1950s[22] but has recently attracted increased interest due to the successful efforts of several groups to improve the control over the NCA polymerization through reduced temperature,[23–25] reduced pressure,[24,26,27] or the employment of specific initiators.[14,28–36] Hence, polypeptides with narrow polydispersities, defined architectures, and end-groups are now accessible via NCA polymerization opening new avenues for material design. One important aspect that has yet to be explored in detail is the understanding of NCA copolymerization both in terms of reaction kinetics and chain microstructure.

The random copolymerization of two amino acid NCAs has been explored in several early publications (reviewed elsewhere[37]) wherein the relative reactivity of the two NCAs was determined through their copolymerization parameters. Progress of the reactions was generally monitored by CO_2 detection[38,39] or infrared spectroscopy;[40,41] however, both methods are not able to distinguish between different amino acid NCAs and hence detailed kinetic information of the consumption of each amino acid in the copolymerization remained elusive. Nuclear magnetic resonance (NMR) (^{1}H-NMR or ^{15}N-NMR) has also been used to study amino acid NCA polymerization kinetics.[42–44] While it is able to distinguish between certain amino acids, it is limited by the fact that it only allows the study of small numbers of monomers due to significant overlap of signals in more complex copolymerization. To address this, we have recently reported a procedure through which we were able to follow the

Additional Supporting Information may be found in the online version of this article.

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consumption of two amino acid NCAs, Glu(Bzl)-NCA and Lys(Z)-NCA, simultaneously during the polymerization.[45] In the present study, we have taken the next step in complexity in our investigation and report on a detailed kinetic study of an NCA terpolymerization. Firstly, we employ our previously developed method to follow amino acid NCA consumption of a more complex mixture containing three NCA monomers, allowing direct access to amino acid NCA kinetics in a terpolymerization for the first time. Secondly, we determine copolymerization parameters for the amino acid NCAs involved in the terpolymerization with the aim to establish relative reactivities of our monomers and evaluate the possibility to predict the composition of terpolymers from binary copolymerizations.

Currently, only a small number of publications investigating the random copolymerizations of more than two amino acid NCA is available. Sommerdijk, Heise, and coworkers prepared Glu(Bzl)/Asp(Bzl)/Ala[46] and Glu(Bzl)/Lys(Z)/Ala[47] terpolymers to control and study biomineralization. Yamamoto et al. prepared a polypeptide from 18 different amino acid NCAs.[48] In these studies, only the overall composition of the final polymers was analyzed, no information on the copolymer kinetics or the chain structure was obtained. Only two reports have studied NCA terpolymerizations in more detail. Hull and Kricheldorf investigated the copolymerization of Gly-NCA/Leu-NCA/Val-NCA and Gly-NCA/Val-NCA/γ-methyl-Glu-NCA with ^{15}N-NMR spectroscopy and thus determined the copolymerization parameters and the average homogenous sequence length of the terpolymers.[44] Wamsley et al. investigated the terpolymerization of Leu-NCA/Asp(Bzl)-NCA/Val-NCA by determining the copolymerization parameters of the binary mixtures (with ^{1}H-NMR spectroscopy) and comparing the experimentally obtained terpolymer composition with the predicted composition.[43] No kinetic data of the progress of a terpolymerization is available to date.

In this work, we first study the kinetics of the copolymerization of Glu(Bzl)-NCA/Tyr(Bzl)-NCA (E/Y) and Lys(Z)-NCA/Tyr(Bzl)-NCA (K/Y), two monomer pairs whose copolymerization has not yet been investigated. Polymers consisting of Glu(Bzl)/Lys(Z) have been prepared before[39,40,49] and we have recently reported the copolymerization kinetics for these materials.[45] We then move on to elucidate the kinetics of the terpolymerization of three NCAs, Glu(Bzl)-NCA, Lys(Z)-NCA, and Tyr(Bzl)-NCA to gain insight into the effects of monomer composition on the reaction kinetics and consequently generate an improved understanding of the polymer composition.

EXPERIMENTAL

Materials

Amino acid derivatives were purchased from Bachem. Dry ethylacetate was obtained from Acros and *n*-heptane, 1,1,1,3,3,3-hexafluoroisopropanol, and acetonitrile from Biosolve. Hexylamine, α-pinene, triphosgene, diethylether, and dry dimethyl formamide (DMF) were obtained from Sigma Aldrich.

NCA Synthesis

Protected amino acids were converted into NCAs as previously described.[45] Details for the preparation of each amino acid NCA can be found in the literature for Glu(Bzl)-NCA and Lys(Z)-NCA[45] and in the Supporting Information for Tyr(Bzl)-NCA.

NCA Polymerization

Polymerization of the amino acid NCAs was carried out with a total concentration of 2 M. The relatively high concentration was chosen to allow the acquisition of data at relatively low conversion. The reaction was initiated with n-hexylamine in dry DMF under N_2 atmosphere in a Schlenk tube according to the literature procedures.[45] The initiator concentration (3.8 μM) was constant for all the reactions. The polymerization was stirred at room temperature until completion. Samples were taken for high-performance liquid chromatography (HPLC) analysis at periodic intervals under N_2 flow and directly subjected to the quenching procedure as described in the HPLC section below. After complete conversion, the final polymer was precipitated in diethylether, washed with ether, and dried in a vacuum oven at 40 °C over night. To obtain polymers for the polymer composition analysis at low conversion, separate reactions were used that were stopped after 20 min by adding 700 μL of the reaction solution to 500 μL of the quenching solution. After addition of 2 mL ethyl ether, the precipitate was filtered and washed with 10 mL diethylether before drying the product at 40 °C overnight.

NMR

The polymers were dissolved in deuterated trifluoroacetic acid (TFA). NMR spectra were acquired on a Varian Mercury 400 Autosampler using automatic acquisition protocols. The spectra were analyzed using MestReNova (v 7.1.1). Peaks from the amino acid parts of the polymer and not from the protection groups were used to determine the copolymer composition as TFA can be expected to cause removal of some protection groups. Peak fitting was performed after baseline correction with a Bernstein polynomial fit. To fit the peaks, component peaks were added manually followed by automatic fitting protocols of the analysis software. This process was repeated until the envelope of the component peaks matched the spectrum and a minimal residual error was obtained.

Mass Spectrometry (MS)

The mass of Tyr(Bzl)-NCA was confirmed with a Voyager DE-STR matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer (Applied Biosystems). Tyr(Bzl)-NCA was dissolved in THF at 1 mg ml^{-1}. Trans-2-[3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene] malononitrile (8 mg in 20 μL THF) was used as matrix and sodium hexadecanesulfonate (1 mg in 200 μL THF) was used as salt. About 0.3 μL of a mixture consisting of salt, matrix, and sample (1:4:4) were spotted on the sample plate and analyzed using a 337 nm laser with a frequency of 20 Hz and a voltage of 25 kV. The composition of the quenched Tyr(Bzl)-NCA was analyzed with liquid chromatography–MS (LC–MS) as described previously for other amino acid NCAs.[45]

Gel Permeation Chromatography (GPC)

GPC of the polymers was performed in 1,1,1,3,3,3-hexafluoroisopropanol containing 3 g l^{-1} trifluoroacetate. The GPC

was equipped with a Waters 1515 Isocratic HPLC pump, a Waters 2414 refractive index detector (40 °C), a Waters 2707 autosampler, a PSS PFG guard column followed by two PFG-linear-XL columns (8 × 300 mm, 7 μ) in series (at 40 °C). To calculate the relative molecular weights, polymethyl methacrylate standards with M_P of 580 Da up to 7100 kDa (Polymer Laboratories) were used.

Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectra were measured on a Perkin Elmer FTIR Spectrometer equipped with a universal sample accessory. A scan resolution of 1 cm^{-1} was used and 10 scans were averaged for each measurement. The liquid samples from the polymerization reactions were deposited onto the crystal without any further sample modification. The spectra were analyzed with Varian Resolutions 4.0 to obtain peak areas for the signals around 1785 cm^{-1} (C═O of NCA) and 1720 cm^{-1} (C═O of protection group). The C═O peak of the protection groups was used as internal standard; the reported data is the ratio of the peak areas at 1785 cm^{-1}/1720 cm^{-1}.

Kinetic Analysis (HPLC)

For quantification of the remaining monomer in the polymerization mixture, a recently described procedure was used.[45] In short, 50 μL of the reaction mixture were quenched in 950 μL of an acidic acetonitrile/water solution. This converts the NCAs back into the original amino acids which were subsequently separated by RP-HPLC via gradual elution with acetonitrile/water using an Agilent Zorbax SB-C18 column in an Agilent 1100 Series chromatograph. The peaks were identified and quantified using calibration curves of the respective individual amino acid NCAs. More details and the calibration curves for Glu(Bzl) and Lys(Z) can be found in the literature.[45] For Tyr(Bzl), validation of the peak position and the corresponding calibration plot can be found in the Supporting Information. The kinetic experiments were performed at least twice for each condition. Both repeats displayed similar values and the same overall trend of the kinetic curve; the data presented in the figures are datasets of one experiment for each condition.

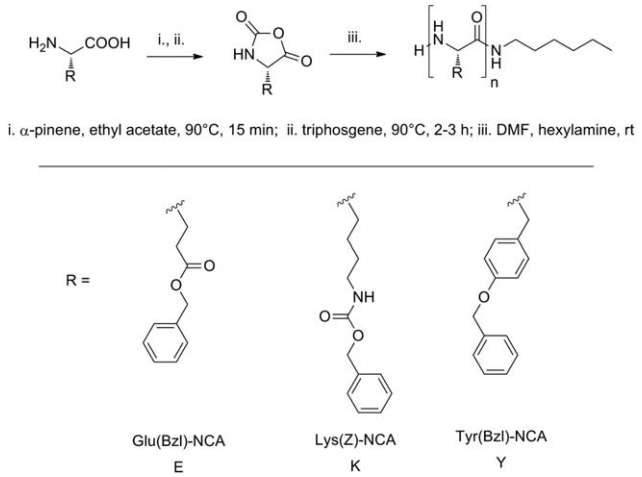
Determination of Copolymerization Parameters

To determine the instantaneous copolymerization parameters for the Glu(Bzl)/Tyr(Bzl) and the Lys(Z)/Tyr(Bzl) system, copolymerizations at different NCA ratios (1:1; 11:9; 3:2; 13:7; 7:3; 3:1, and 4:1) were carried out for 20 min. About 700 μL of the reaction were then quenched by addition to 1 mL 15% HCl in acetonitrile/water (1:1). About 2 mL diethylether was added and the precipitate was filtered and washed with 10 mL diethylether before drying in the vacuum oven at 40 °C overnight. The resulting polymer was then analyzed by NMR as described above.

RESULTS AND DISCUSSION

Copolymerization Kinetics of Glu(Bzl)/Tyr(Bzl) and Lys(Z)/Tyr(Bzl)

To study the kinetics of a terpolymer, a detailed understanding of the copolymerization of the three involved monomers



SCHEME 1 Reaction scheme for the synthesis of NCAs and polypeptides from Glu(Bzl), Lys(Z), and Tyr(Bzl).

in a binary mixture is essential. Hence, before studying the copolymerization of Glu(Bzl), Lys(Z), and Tyr(Bzl), we first compare the kinetics of the copolymerization of the binary systems Glu(Bzl)/Lys(Z), Glu(Bzl)/Tyr(Bzl), and Lys(Z)/Tyr(Bzl). As we have recently reported the copolymerization kinetics for the first monomer pair, Glu(Bzl)/Lys(Z), to establish the procedure for the kinetic analysis of amino acid NCA polymerizations[45] only data for Glu(Bzl)/Tyr(Bzl) and Lys(Z)/Tyr(Bzl) will be presented here, even though comparisons with the Glu(Bzl)/Lys(Z) pair will be drawn, as well.

The procedure used for the preparation of the NCA monomers and the NCA polymerization is shown in Scheme 1. For the polymerization, different monomer ratios were used, keeping the overall NCA concentrations constant at 2 M. During the reaction, samples were drawn to follow the consumption of each individual amino acid NCA using our recently established procedure.[45] In brief, this consisted of the quenching of samples taken at specific timepoints in an acidic solution to convert non-reacted amino acid NCAs back into the original amino acids. These samples were subsequently analyzed by HPLC which allowed simultaneous quantitative analysis of the amino acid NCA concentration in the feed solution over time. Since this procedure's validity was only demonstrated for Gly(Bzl)-NCA and Lys(Z)-NCA, here we first verified that it can be equally applied to Tyr(Bzl). Supporting Information Figure S1 shows that acidic quenching converts Tyr(Bzl)-NCA back into the original protected amino acid. The small shift in the HPLC retention time between the original amino acid and the quenched NCA is due to a small concentration dependence of the peak position that has been noted previously.[45] Supporting Information Figure S2 shows the HPLC calibration graph used to determine the monomer concentration. For Glu(Bzl) and Lys(Z), the calibrations reported in our previous study[45] were used. The ability of the HPLC method to distinguish Tyr(Bzl) from either Glu(Bzl) or Lys(Z) is shown in Supporting Information Figure S3 where the peaks are well separated from each other. The small peak at approximately 17

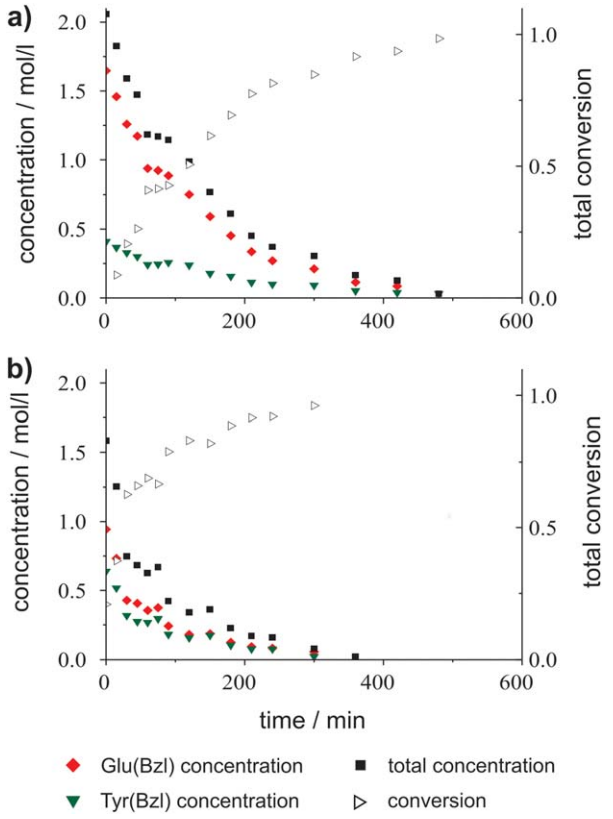


FIGURE 1 Individual and total consumption of Glu(Bzl)-NCA and Tyr(Bzl)-NCA during polymerization at two different monomer ratios and overall conversion of NCAs during the reaction. (**a**) Glu(Bzl):Tyr(Bzl) = 3:1; (**b**) Glu(Bzl):Tyr(Bzl) = 1:1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

min in the Glu(Bzl)/Tyr(Bzl) chromatogram in Supporting Information Figure S3 was previously allocated to low amounts of Glu that were produced through deprotection of Glu(Bzl) in the acidic quenching solution.[45]

Figures 1 and 2 show the progress of the Glu(Bzl)/Tyr(Bzl) and Lys(Z)/Tyr(Bzl) copolymerizations for two monomer ratios (X:Tyr(Bzl) = 3:1 and 1:1). Notably, copolymerizations with a Tyr(Bzl)-NCA concentration of 1.5 M result in the formation of a gel within minutes after initiation, making the monitoring of copolymerizations with high Tyr(Bzl)-NCA content impossible. Hence, data was only collected for reactions in which the Tyr(Bzl)-NCA concentration was 1 M or less.

The measured starting monomer concentrations match well with the theoretical values for all four reactions. In addition, the relative decrease of the total monomer concentration (determined by the HPLC method) follows a very similar trend to that observed by IR (see Supporting Information Fig. S4), confirming the validity of the HPLC measurement as an approach to follow these copolymerizations. The polymerizations reach full conversion after approximately 7–10 h, a timeframe comparable to that of the Glu(Bzl)/Lys(Z) system we investigated previously.[45]

When fitting the kinetic data obtained from these reactions to rate law equations, linear correlations were found when a first order rate law equation was used (Supporting Information Fig. S5). This enabled the determination of rate law constants (k) for the whole reaction as well as for the consumption of each individual monomer. Table 1 gives values for k for the two systems reported here as well as for the Glu(Bzl)-NCA/Lys(Z)-NCA system which were calculated from data we reported previously.[45] From this data it is evident that the reaction rate for one amino acid NCA not only depends on the second monomer but also on the relative concentrations of the two monomers. While Glu(Bzl)-NCA polymerizes faster than Tyr(Bzl)-NCA if E:Y = 3:1, the reaction rates are similar for both monomers when E:Y = 1:1. In contrast, for both ratios of Lys(Z)-NCA and Tyr(Bzl)-NCA, both monomers polymerize with similar rates. When paired with Glu(Bzl)-NCA, the rate of Lys(Z)-NCA incorporation into the polymer is significantly lower than that of Glu(Bzl)-NCA at all monomer ratios. Generally, it can be concluded that Glu(Bzl)-NCA reacts faster than the other two NCA monomers and is thus likely to be preferentially incorporated into the polymer. In contrast, Lys(Z)-NCA and Tyr(Bzl)-NCA have similar reaction rates and their copolymerization can be

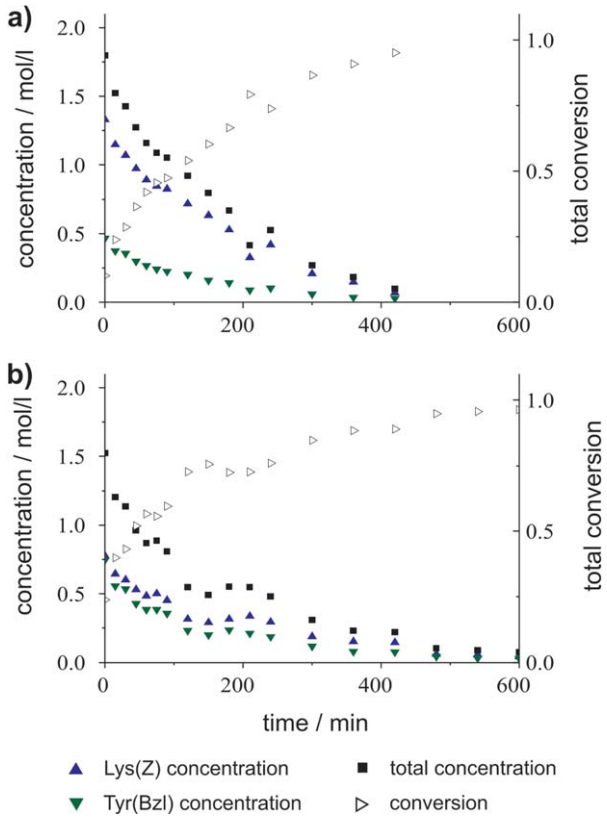


FIGURE 2 Individual and total consumption of Lys(Z)-NCA and Tyr(Bzl)-NCA during polymerization at two different monomer ratios and overall conversion of NCAs during the reaction. (**a**) Lys(Z):Tyr(Bzl) = 3:1; (**b**) Lys(Z):Tyr(Bzl) = 1:1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1 First Order Reaction Rate Constants for the Consumption of NCAs in Binary Copolymerizations[a]

Reaction[b]	NCA 1[c,d] × 10^{-3} s^{-1}	NCA 2 [c,d] × 10^{-3} s^{-1}	Total NCA[d] × 10^{-3} s^{-1}
E:Y = 3:1	7.1 ± 0.1	5.4 ± 0.2	6.7 ± 0.1
E:Y = 1:1	9.0 ± 0.6	9.5 ± 0.7	9.1 ± 0.5
K:Y = 3:1	6.4 ± 0.3	6.5 ± 0.2	6.4 ± 0.3
K:Y = 1:1	4.5 ± 0.2	5.2 ± 0.2	4.8 ± 0.2
E:K = 3:1	7.2 ± 0.2	3.8 ± 0.1	5.9 ± 0.1
E:K = 1:1	5.4 ± 0.2	3.8 ± 0.2	4.5 ± 0.2
E:K = 1:3	11.7 ± 0.2	7.0 ± 0.2	7.9 ± 0.2

[a] Initiator concentration was 3.8 μM.
[b] E = Glu(Bzl)-NCA; K = Lys(Z)-NCA; Y = Tyr(Bzl)-NCA.
[c] First and second amino acid NCA shown in the labels in the left hand column.
[d] Errors originate from the statistical fitting of a linear regression through one kinetic curve

expected to yield a more even distribution of monomer units in the final polymer.

Copolymerization Parameters

To predict the composition of a terpolymer, theoretical models such as the Alfrey–Goldfinger equations have been developed.[50] These models require knowledge of the copolymerization parameters of the three monomers from binary reactions. We have recently reported the copolymerization parameters for the Glu(Bzl)/Lys(Z) system,[45] where $r_{EK} = 1.87 \pm 0.31$ and $r_{KE} = 0.52 \pm 0.11$ according to the Kelen–Tüdös (KT) method. Here, we used the same procedure to determine the copolymerization parameters for the Glu(Bzl)/Tyr(Bzl) and Lys(Z)/Tyr(Bzl) systems.

Copolymerizations were carried out at seven different monomer ratios. Due to the previously mentioned issues with gelation at Tyr(Bzl)-NCA concentrations higher than 1 M, the concentration range was varied at 0.2 M intervals between 1 M and 0.4 M Tyr(Bzl)-NCA, while the concentration of the second NCA was increased to give a constant total NCA concentration of 2 M. The copolymerizations were stopped at low conversion (20 min reaction time) and NMR spectra of the isolated copolymers were used to determine the relative amount of each amino acid in the polypeptide. Typical ^{1}H-NMR spectra for these systems are shown in Supporting Information Figures S6 and S7. Glu(Bzl) was identified using the CH peak at 4.6 ppm, Lys(Z) by the CH peak at 4.9 ppm, and Tyr(Bzl) was identified using the CH_2 peak between 2.5 and 3 ppm. Using these experimental polymer compositions, the copolymerization parameters in Tables 2 and 3 were calculated via the Fineman–Ross (FR) and KT methods. The numeric data obtained is provided in Supporting Information Tables S1 and S2; and the regression lines produced from these calculations are shown in Supporting Information Figures S8 and S9. Due to the low Tyr(Bzl)-NCA concentration, the linear regressions obtained for these experiments display a lower R^2 than the previously reported Glu(Bzl)/Lys(Z) system. For the Lys(Z)/Tyr(Bzl) system in particular, the marker signals of Tyr(Bzl) in the NMR spectrum suffer from the proximity of other Lys(z) related peaks, thus making the quantification more prone to errors which becomes more pronounced as the Tyr(Bzl) concentration decreases. Hence, twice as many data points were collected for the Lys(Z)/Tyr(Bzl) system to improve the statistical fidelity of the determination of the copolymerization parameter.

TABLE 2 Copolymerization Parameters for Glu(Bzl)-NCA (r_{EY}) and Tyr(Bzl)-NCA (r_{YE}) Determined From ^{1}H-NMR Data and Calculated Using Two Different Models

	r_{EY}	r_{YE}	$r_{EY} \times r_{YE}$
Fineman–Ross	1.33 ± 0.07	0.04 ± 0.10	0.05
Kelen–Tüdös	1.39 ± 0.29	0.02 ± 0.11	0.02

For Glu(Bzl)-NCA/Tyr(Bzl)-NCA, the parameter r_{YE} displays a large uncertainty due to the relatively large fitting error. Nevertheless, it is clear that r_{EY} is significantly larger than r_{YE}. This indicates that Glu(Bzl) has a much higher propensity to be incorporated into the copolymer than Tyr(Bzl), making Glu(Bzl) the most reactive of the three amino acid NCAs. In contrast to the Glu(Bzl)/Lys(Z) system, however, where the product of $r_{EK} \times r_{KE}$ was only slightly less than unity, the product of $r_{EY} \times r_{YE}$ is near zero, indicating that the copolypeptide obtained from this reaction does not follow a random sequence distribution. This is also shown in Figure 3(a), where the plot of the mole fraction in the polymer versus the initial mole fraction in the feed shows a composition drift away from that of an ideal copolymer toward a higher Glu(Bzl) content.

In the Lys(Z)/Tyr(Bzl) system, Tyr(Bzl) is the more reactive monomer (Table 3), although the difference is far less pronounced than for the Glu(Bzl)/Tyr(Bzl) system. This indicates that Lys(Z) is the least reactive of the three NCAs. In contrast, to the Glu(Bzl)/Tyr(Bzl) system, however, the product of $r_{KY} \times r_{YK}$ is much closer to unity, suggesting that the sequence in the polypeptide is more random. This is confirmed in Figure 3(b), where no significant composition drift was observed. Instead, the data matches closely with the line indicating the situation for an ideal random copolymerization. The copolymerization parameters match well with the conclusions made on the basis of the HPLC-based kinetic data and the rate constants. Both methods indicate the same relative reactivities for the three monomers and suggest a more random monomer sequence distribution for the Lys(Z)/Tyr(Bzl) copolymer.

TABLE 3 Copolymerization Parameters for Lys(Z)-NCA (r_{KY}) and Tyr(Bzl)-NCA (r_{YK}) Determined From ^{1}H-NMR Data and Calculated Using Two Different Models

	r_{KY}	r_{YK}	$r_{KY} \times r_{YK}$
Fineman–Ross	0.90 ± 0.09	1.43 ± 0.24	1.29
Kelen–Tüdös	0.86 ± 0.29	1.33 ± 0.23	1.14

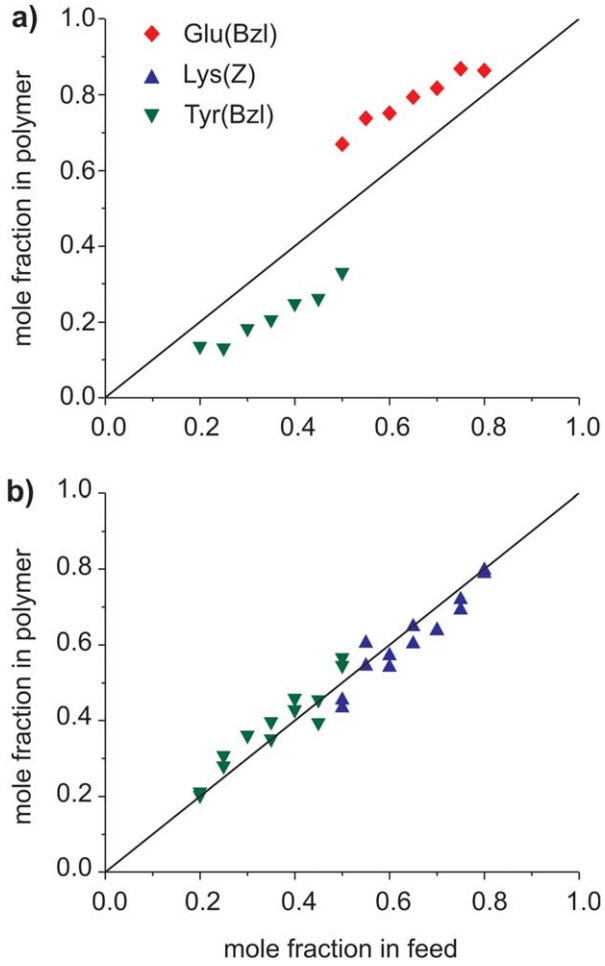


FIGURE 3 Copolymer composition (obtained from ^{1}H-NMR) as function of the initial mole fraction of the NCAs in the feed. The *line* represents the case of an ideal copolymerization. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

It is well known that various factors including electronic and steric properties of the NCA and the initiator as well as solvent polarity have pronounced effects on the reaction kinetics of NCA polymerizations.[37,51] Under the conditions of the present study, the incorporation of Glu(Bzl)-NCA is always favored, regardless of the nature of the amino acid on the end of the polymer. This indicates that the reaction kinetics are mostly governed by the properties of the monomer as opposed to those of the active chain end of the polymer. As a primary amine initiator was used, it can be assumed that the normal amine mechanism (NAM) is the main propagation mechanism for the polymerization.[37] In this mechanism, the free amine of the polymer chain performs a nucleophilic attack on the carbonyl group of the NCA. The mechanistic steps involved in the propagation of the NAM are very complex, involving a series of transition states and proton rearrangements.[52] While we can conclude that the nature of the different reactivities observed here must lie in the susceptibility of the NCA carbonyl to a

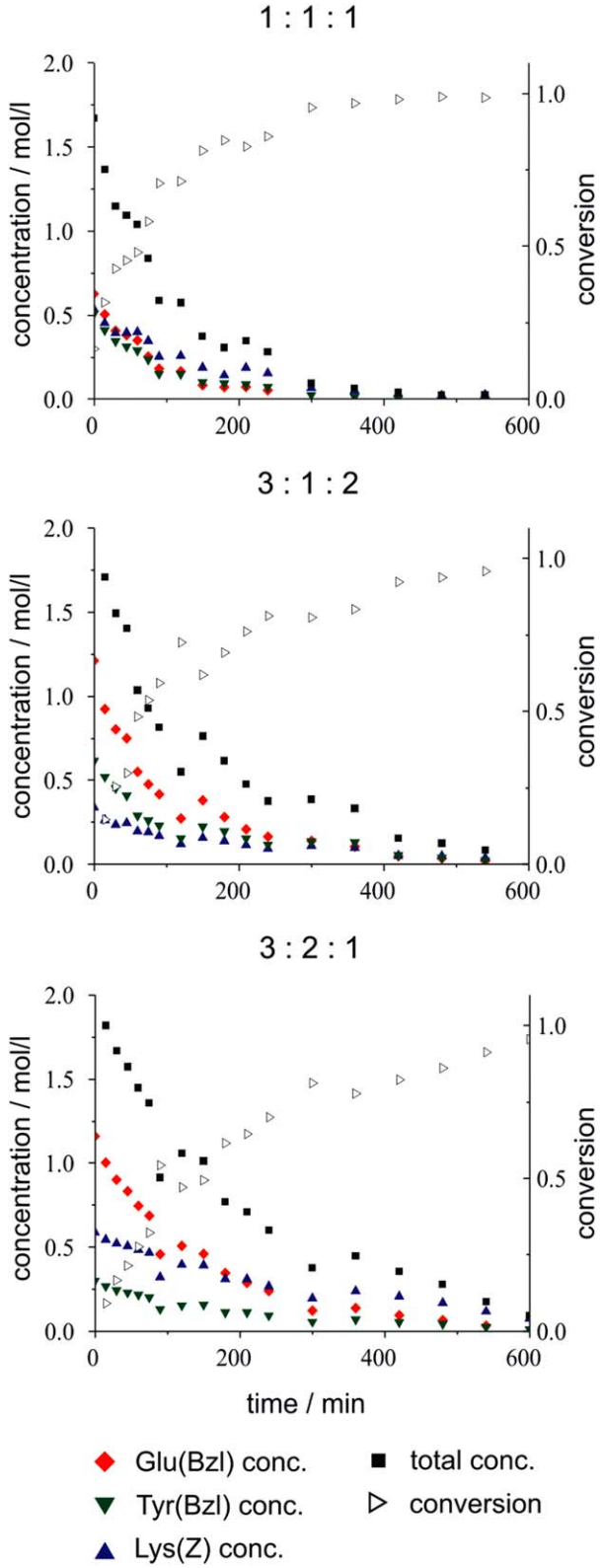


FIGURE 4 Individual and total consumption of Glu(Bzl)-NCA, Lys(Z)-NCA, and Tyr(Bzl)-NCA during the terpolymerization at selected monomer ratios and overall conversion of NCAs during the reaction. Data for all seven monomer ratios can be found in the Supporting Information (Figure S11). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 4 First Order Reaction Rate Constants for the Consumption of NCAs in Terpolymerizations with Various Monomer Starting Concentrations[a]

Reaction[b]	Glu(Bzl)-NCA[c] $\times 10^{-3}$ s^{-1}	Lys(Z)-NCA[c] $\times 10^{-3}$ s^{-1}	Tyr(Bzl)-NCA[c] $\times 10^{-3}$ s^{-1}	Total NCA[c] $\times 10^{-3}$ s^{-1}
E:K:Y = 1:2:3	14.0 ± 0.6	6.2 ± 0.3	8.5 ± 0.4	7.7 ± 0.4
E:K:Y = 1:3:2	8.4 ± 0.4	5.4 ± 0.2	6.8 ± 2.2	6.2 ± 2.3
E:K:Y = 2:1:3	8.6 ± 0.5	4.6 ± 0.6	7.3 ± 0.5	8.9 ± 0.7
E:K:Y = 2:3:1	6.8 ± 0.2	3.9 ± 0.3	4.9 ± 0.2	4.9 ± 0.3
E:K:Y = 3:1:2	6.9 ± 0.3	3.5 ± 0.2	5.1 ± 0.4	5.4 ± 0.3
E:K:Y = 3:2:1	6.1 ± 0.2	2.9 ± 0.2	4.5 ± 0.2	4.5 ± 0.2
E:K:Y = 1:1:1	10.7 ± 0.6	6.1 ± 0.3	9.7 ± 0.4	8.3 ± 3.1

[a] Initiator concentration was 3.8 μM.
[b] E = Glu(Bzl)-NCA; K = Lys(Z)-NCA; Y = Tyr(Bzl)-NCA.
[c] Errors originate from the statistical fitting of a linear regression through one kinetic curve.

nucleophilic attack, to date there is no systematic study available that would provide more insight into the effect of the side group on the monomer reactivity.

Terpolymerization Kinetics

The terpolymerization of Glu(Bzl), Lys(Z), and Tyr(Bzl) was studied in the same manner as the binary systems. The three NCAs were polymerized at different ratios, given subsequently in the order Glu(Bzl):Lys(Z):Tyr(Bzl). The total NCA concentration was kept constant at 2 M. Samples were taken periodically during the polymerizations and analyzed by HPLC. After completion of the reaction, the isolated polymers were analyzed by GPC (Supporting Information Fig. S10). The GPC data shows that the polymers were monomodal, suggesting that they are indeed copolymers and not polymer blends. Small shoulders in the traces are attributed to the interference introduced by drawing samples.

Examples of the kinetic curves obtained from the HPLC measurements are shown in Figure 4; the data for all seven monomer ratios can be found in Supporting Information Figure S11. As with the binary systems, the measured starting concentrations are similar to the expected theoretical values with small deviations being attributed to experimental limitations due to sample preparation and measurement. Full conversion was reached after approximately 7–8 h.

The reaction rate constants of the terpolymerizations were determined by fitting the data to a first order rate law (Supporting Information Fig. S12). While linear trends were observed, it should be noted that the fit for the binary copolymerizations appeared to be better, suggesting that the addition of a third amino acid decreases the precision of the experimental procedure. The values for the rate constants are given in Table 4. For all reactions, the rate constants for Glu(Bzl)-NCA are highest, followed by those of Tyr(Bzl)-NCA. This reflects the same trend in monomer reactivity suggested by the binary copolymerizations. As with the binary reactions, a concentration dependence of the rate constant can be observed; with a few exceptions, the rate constants decrease as the initial concentration of the respective monomer is reduced. We propose that this observation is in parts due to the relative probability of the monomer to collide with an active polymer chain end.

Terpolymer Composition

To investigate the composition of the terpolymers, ^{1}H-NMR of polypeptides obtained after 20 min reaction were carried out as described above. A typical ^{1}H-NMR spectrum of a terpolymer is shown in Supporting Information Figure S13. Curve fits to the backbone CH of Glu(Bzl) and Lys(Z) and the

TABLE 5 Comparison of the Experimental and Predicted Terpolymer Compositions

	Experimental Polymer Composition[a]			Predicted Polymer Composition[b]		
Feed Composition (E:K:Y)	E	K	Y	E	K	Y
1:1:1	0.60	0.16	0.25	0.57	0.19	0.24
1:2:3	0.46	0.18	0.35	0.48	0.18	0.35
1:3:2	0.44	0.31	0.26	0.43	0.31	0.26
2:1:3	0.62	0.07	0.31	0.60	0.08	0.32
2:3:1	0.55	0.34	0.11	0.54	0.32	0.14
3:1:2	0.68	0.09	0.22	0.69	0.08	0.22
3:2:1	0.70	0.20	0.10	0.68	0.20	0.13

[a] From NMR spectra after 20 min reaction time (~10%–30% conversion).
[b] Calculated using the Alfrey–Goldfinger equations and the copolymerization parameters from the binary polymerization.

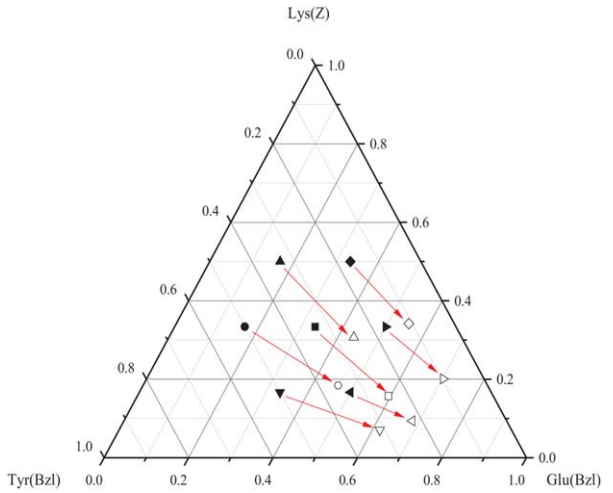


FIGURE 5 Ternary diagram showing the composition of the feed (*solid symbols*) and the polymer (*open symbols*) obtained after polymerization for 20 min. Corresponding feed/polymer compositions were given the same symbol-shape. *Arrows* highlight the direction of the composition shift between the feed and the polymer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CH_2 of the tyrosine side chain allowed an estimation of relative composition of the terpolymers (Table 5).

The ability of the copolymerization parameters determined from binary copolymerizations to predict the composition of the terpolymer was explored using the Alfrey–Goldfinger equation. Here, the change in NCA concentrations, d[E], d[K], and d[Y], is related directly to the amount of amino acid incorporated into the polymer. This can be expressed as a function of the initial NCA concentrations ([E], [K], and [Y]) and their copolymerizations parameters:

$$
\begin{aligned}
&d[\mathrm{E}]:\mathrm{d}[\mathrm{K}]:\mathrm{d}[\mathrm{Y}]\\
&=[\mathrm{E}]\left(\frac{[\mathrm{E}]}{r_{\mathrm{YE}}r_{\mathrm{KE}}}+\frac{[\mathrm{K}]}{r_{\mathrm{KE}}r_{\mathrm{YK}}}+\frac{[\mathrm{Y}]}{r_{\mathrm{YE}}r_{\mathrm{KY}}}\right)\left([\mathrm{E}]+\frac{[\mathrm{K}]}{r_{\mathrm{EK}}}+\frac{[\mathrm{Y}]}{r_{\mathrm{EY}}}\right):\\
&[\mathrm{K}]\left(\frac{[\mathrm{E}]}{r_{\mathrm{EK}}r_{\mathrm{YE}}}+\frac{[\mathrm{K}]}{r_{\mathrm{EK}}r_{\mathrm{YK}}}+\frac{[\mathrm{Y}]}{r_{\mathrm{YK}}r_{\mathrm{EY}}}\right)\left([\mathrm{K}]+\frac{[\mathrm{E}]}{r_{\mathrm{KE}}}+\frac{[\mathrm{Y}]}{r_{\mathrm{KY}}}\right):\\
&[\mathrm{Y}]\left(\frac{[\mathrm{E}]}{r_{\mathrm{EY}}r_{\mathrm{KE}}}+\frac{[\mathrm{K}]}{r_{\mathrm{KY}}r_{\mathrm{EK}}}+\frac{[\mathrm{Y}]}{r_{\mathrm{EY}}r_{\mathrm{KY}}}\right)\left([\mathrm{Y}]+\frac{[\mathrm{E}]}{r_{\mathrm{YE}}}+\frac{[\mathrm{K}]}{r_{\mathrm{YK}}}\right)
\end{aligned}
$$

To calculate the predicted terpolymer compositions, the copolymerization parameters obtained from the KT method were used because they are more robust to the effect of single outlying data points.[43,45] The results obtained from this approach are given in Table 5. The predicted values are in very good agreement with the experimental data, thus validating the copolymerization parameters calculated for the binary systems and supporting the experimental results for the terpolymer composition.

To visualize the correlation between the mole fraction in the feed and the experimentally observed terpolymer composition, a ternary diagram is presented in Figure 5. It becomes immediately evident that all polypeptides have different compositions from that of the initial feed concentrations. In addition, the initial polymer composition is shifted directly toward higher Glu(Bzl) content in all reactions. This is consistent with the observations in the kinetic experiments where Glu(Bzl)-NCA was identified as the most reactive monomer. The preferential incorporation of Glu(Bzl) at the start of the reaction suggests that the starting end of the polypeptides contain longer Glu(Bzl) sequences than the rest of the polymer. Thus, the observed shift in the initial polypeptide composition during the terpolymerization can be well rationalized using the kinetic data and the copolymerization parameters of the NCAs.

CONCLUSIONS

This study demonstrates for the first time that it is possible to determine the kinetics of individual amino acid NCAs during an NCA terpolymerization. We have employed this method for the elucidation of the reaction kinetics of three amino acid NCAs, Glu(Bzl)-NCA, Lys(Z)-NCA, and Tyr(Bzl)-NCA; and presented copolymerization parameters and reaction rate constants for these polymerizations. The kinetic data provides a higher level of understanding of NCA copolymerizations in complex mixtures, paving the way for a better structure/property understanding. We believe that the insight and control gained from this approach will contribute to the design of well-defined copolypeptides for applications as drugs and responsive biomaterials.

ACKNOWLEDGMENTS

We are grateful to Anneke Delsing for help with the HPLC measurements and to Joost van Dongen for the LC-MS measurements. MZ acknowledges financial support through a Marie Curie fellowship (FP7-PEOPLE-2011-IEF 299280). AH is a SFI Stokes Senior Lecturer (07/SK/B1241).

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