Accelerated Polypeptide Synthesis *via* *N*-carboxyanhydride Ring Opening Polymerization in Continuous Flow

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ABSTRACT

In nature, polypeptide-based materials are ubiquitous, yet their synthetic production is hampered by high cost, limited scalability and often stringent reaction conditions. Herein an elegant approach is presented for NCA ROP of *N* ε-benzyloxycarbonyl L-Lysine (ZLL) and γ-benzyl-L-glutamate (BLG) NCA in continuous flow. The polymerization is initiated by primary amine intiators using *N,N*-dimethylformamide (DMF) as solvent. Carrying out the reaction in a silicon microflow reactor sped up the rate of ROP (92% conversion in 40 minutes in flow as opposed to 6 hours in batch) due to highly efficient permeation of CO_2 through the reactor tubing. Our polymerization strategy provides a facile, scale-up friendly alternative to traditional batch mode polymerization and has the capability of streamlining NCA ROP.

Keywords: polypeptides; NCA; ring-opening polymerization; flow chemistry

Peptide based materials are gaining increasing interest in material design as well as in medicinal fields.[1-4] Their wide utility stems from their biocompatible and biodegradable character, in addition to the peptide's capacity to generate secondary structures.[5, 6] Polypeptides are commonly synthesized in one of three ways: solid phase peptide synthesis (SPPS), recombinant DNA synthesis,[7, 8] or *N*-carboxyanhydride ring opening polymerization (NCA ROP).[9-11] Synthetic sequence-defined polypeptides are often acquired *via* (automated) SPPS. Mijalis *et al.* sped up this synthetic approach with a revolutionary automated continuous flow-based procedure for peptide synthesis based on SPPS, reporting convenient synthesis of up to 30-mer peptides.[12] NCA ROP is however the most expedient and economical method for production of synthetic polypeptides in a multigram scale. An example of industrialized synthesis of polypeptides *via* NCA ROP is glatiramer acetate (tradename Copaxone® for treatment of Multiple Sclerosis), obtained in batch mode [13, 14] Interestingly, the use of flow chemistry as a synthetic method is a strong emerging trend in the pharmaceutical industry; in fact, the Food and Drug Administration has been strongly stimulating the application of flow technology in the context of active pharmaceutical ingredients.[15] In response, the biopharmaceutical industry is beginning to recognize the advantages this technique can offer, and is adopting knowledge already present in academic labs, food, petrochemical and chemical industries.[16] Flow chemistry thus presents itself as a rational approach to further consider in the NCA ROP-polypeptide field.[15, 17, 18] While recent work on continuous flow for peptide synthesis *via* NCA ROP has focused mainly on the formation of di- and tripeptides,[19-21] a flow process to synthesize NCA monomers has been recently realized by the Fuse group.[22] Challenges remain to translate the NCA ROP reaction to flow however, as so far only a few examples exist in the literature. Honda *et al.* were the first to synthesize polypeptides *via* ROP in a continuous flow fashion in 2005.[23] These flow-synthesized polypeptides had a lower dispersity than those formed in a classical batch approach. Improvements on the reactor design were published in a follow-up study in which upscaling was also highlighted for production of polypeptides *via* continuous flow.[24] Nevertheless, the polymerization relied on the activated monomer mechanism (AMM) *via* initiation with tertiary

amines instead of the often desired normal amine mechanism (NAM). Additionally, the reaction did not appear to reach full conversion, a detrimental observation given the high synthetic cost of NCA monomers.

In general, amine-initiated NCA ROP proceeds at a reduced rate compared to the anionic AMM and may require several days to reach high conversion in *N,N*-dimethylformamide (DMF) systems, depending on the mechanism of polymerization and initiator chosen. Batch approaches to increase reaction rate include the use of nitrogen flow[25, 26] and the intermittent removal of CO_2 using high vacuum techniques as the reaction proceeds.[27, 28] Most recently, increases in the rate of the ROP have been achieved through exploitation of α-helical macrodipoles to induce cooperative polymerization in helicogenic solvents such as dichloromethane, chloroform and 1,2-dichloroethane.[29-31] Additionally, organocatalysts may be introduced to increase the rate of polymerization. Zhang and coworkers proposed the introduction of imidazolium hydrogen carbonates to accelerate the rate of reaction in DMF, accessing linear and cyclic topologies through the dosage of organocatalyst, and optional addition of amine initiator.[32] Hadjichristidis and coworkers recently reported a substantial increase in polymerization rate afforded by the use of a fluorinated alcohol catalyst in chlorinated solvents.[33] The organocatalyst was found to form multiple cooperative hydrogen bonds, activating the NCA monomers and simultaneously protecting the initiator/growing polymer chain ends, resulting in a polymerization with high activity and selectivity. These recent advances in NCA ring opening polymerization provide an exciting variety of options to access polypeptides with increased speed for a plethora of applications.[11, 34] The demonstrated wide utility of polypeptides warrants the continued scientific interest in simplifying and streamlining their syntheses, and it is our aim to demonstrate that adoption of flow chemistry techniques can further add to the toolbox of effective synthetic strategies. Flow chemistry offers advantageous properties such as more efficient mixing, high surface area and the promise for more convenient scalability. These advantages can result in improved reaction conditions compared with conventional batch NCA ROP *via* NAM. By focusing on NCA ROP *via* the NAM mechanism our work is

distinct from the previous flow reactions performed by Honda *et al.* We fill in the gap left by the previous studies whereby the choice of initiator only allowed for the synthesis of linear and high molecular weight polypeptides. In AMM the basic initiator abstracts the acidic proton from the N-3 position of a monomer, leading to an NCA anion which then performs a nucleophilic attack on another monomer, starting the propagation process, and leading to polypeptides of high molecular weight but reduced functionality, since the polypeptide's C-terminal end group is not functional. The synthesis of more complex polypeptide structures (e.g. star shaped polypeptides) – useful for applications such as drug delivery or antimicrobial agents[35, 36] — is also limited with the AMM choice since these structures often necessitate a multifunctional initiator molecule or terminal reactive handles to direct the formation of complex architecture.[37-39] In this regard the NAM pathway offers more opportunities for functionalization, in addition to also enabling the synthesis of polypeptoids if desired.

Herein we demonstrate a convenient NAM flow approach for the synthesis of polypeptides. Polypeptides were synthesized in reduced timeframes compared to batch. Flow approaches have a strong upscaling potential as shown before for other flow polymerizations.[40, 41] Our method alleviates issues regarding scalability and slow polymerization, providing the potential of streamlining NCA ROP without needing relatively expensive or tedious reagents and equipment. We are convinced that this low-cost system will allow researchers to synthesize relevant polypeptides in a facile manner for applications ranging from the biomedical field to materials development.

The often-observed apparent slow reaction rates of NCA ROP *via* NAM in traditional solvents (i.e. DMF) can be explained when looking at the reaction mechanism depicted in **scheme 1 (A)**. A carbamic acid intermediate is formed upon ring opening by the primary amine initiator. Decarboxylation generates CO_2 and a free amino group, which serves as new nucleophile for further chain propagation. If CO_2 is not removed from the reaction system the polymerization rate will

decrease since the equilibrium is shifted towards the carbamic acid intermediate.[42] Thus, polymerizations can be sped up and intensified by efficient removal of CO_2 which ensures the equilibrium shifts towards decarboxylation and the continuous generation of active amines. The Wooley group studied this behavior in detail by sparging or flowing nitrogen over batch polymerization mixtures at various flow rates.[26] They observed conversions above 90% for the polymerization of γ-benzyl-L-glutamate (BLG) NCA within 2-4 hours depending on the N_2 flow rate.

We hypothesized that a CO_2 permeable flow reactor could ensure even more efficient removal of CO_2 given its very high surface area. Two tubing materials with high CO_2 permeability are commercially available: PTFE AF-2400 and silicone. Given the low-cost nature of the latter and its chemical compatibility with DMF, we investigated the use of silicone tubing for NCA ROP in continuous flow as the more economical solution. The designed flow setup is depicted in **scheme 1 (B)**.

As a reference point, we firstly conducted a comparative batch reaction for NCA ROP in DMF of Nε-benzoxycarbonyl-L-lysine (ZLL) NCA initiated by a norbornene initiator containing primary amine functionality (NB-NH_2 **Figure 1** red squares). NCA ROP *via* NAM with this monomer can be challenging and produces polypeptides that are tedious to characterize due to structural transitions depending on chain length.[43] Yet it is a highly interesting monomer given the strong application of said (co)polypeptides in the biomedical fields.[10, 36, 44, 45] Continuous flow experiments were also carried out with γ-benzyl-L-glutamate NCA to allow more straightforward characterization. A norbornene-containing free amine initiator was chosen to achieve α and ω-functional polypeptides. This functional group enables post-polymerization modifications *via* thiol-ene and inverse electron demand Diels-Alder chemistries,[46, 47] in addition to being readily polymerizable *via* ROMP, allowing the formation of complex structures such as brush polypeptides.[48, 49] As a reference point, the batch reaction (NCA ROP of ZLL) was monitored online with *in situ* attenuated total reflectance Fourier

transform infrared spectroscopy (ATR-FTIR) and was found to be relatively fast in the initial stages, reaching 50% monomer conversion within one hour. However, to reach full conversion, much longer reaction times are required. Size exclusion chromatography (SEC) of the formed polymer revealed a number average molecular weight, M_n of 5300 g · mol^{-1} (**Figure S4**)

Next, we performed the same experiment in our flow reactor. By varying the flow rate of the syringe pump different residence times were screened. From **Figure 1** (green dots) a much higher conversion at shorter timepoints becomes apparent (92% conversion as opposed to 39% in batch at 40 minutes residence/reaction time). This is a remarkable observation and showed a rate increase by a factor of 9.5. The molecular weight for both methods at 40 minutes reaction/residence time is still similar ($M_{n,batch}$ = 5300 g · mol^{-1}, $M_{n,\,flow}$ = 5200 g · mol^{-1}) demonstrating similar chain initiation efficiency. The dispersity of the polymer obtained in flow is also comparable to the one in batch (see discussion below for details). Characterization by matrix assisted laser desorption ionization time of flight (MALDI-TOF) and SEC analysis was performed on these samples as shown in **Figure 2**. Uniform polymers with high end group fidelity are obtained, showing that the process is well controlled, despite the shoulders seen in SEC.

Bimodality of the polymer distribution was seen for poly(lysines) (including batch reactions) and caused the relatively high observable dispersities. Bimodality in SEC traces of these polypeptides with protecting groups has been observed before. A detailed investigation by the Barz group on the cause and origin indicates that this is due to a change in secondary structure of the growing peptide chain.[43] As poly(lysine) grows, a coil-helix transition occurs leading to a different hydrodynamic radius and henceforth elution volume. The transition occurs around a degree of polymerization (DP) of 10-15, also corresponding to our data. At higher conversions, the amount of poly(lysine) in the random coil conformation diminishes, resulting in more monomodal shapes of the polypeptide distributions. Care should therefore be taken in interpreting dispersity data. The continuous flow experiments were also carried out using BLG NCA, as this monomer yields polypeptides which allow

a more straightforward analysis. These results are summarized in **Table 1** (entry 7) and the conversion *vs* residence time profile can be found in the supporting information (**Figure S7**, red diamonds). At 40 minutes residence time a monomer conversion of 92% was reached for the BLG NCA ROP, demonstrating a similar trend to ZLL NCA ROP in flow. The molecular weight distributions indicated again an increase in molecular weight for increasing residence times with a final M_n = 5600 g·mol^{-1} and $Đ$ = 1.4 at 40 minutes residence time (see **Figure S10 A)**. Unfortunately, a bimodal character could still be seen, indicating that secondary structures are likely still formed at this DP range.

When investigating the poly(lysine) polymer distribution *via* MALDI-TOF, shown in **Figure 2B**, the main distribution is composed of polypeptides initiated *via* the NAM with the norbornene initiator. It must be noted that in the lower mass region slight water initiation as well as initiation *via* AMM was observed with MALDI-TOF. The former could be explained by any residual water molecules in the solvent despite flushing the reactor with dry solvent. Initiation *via* AMM can be explained by the fact that simultaneous AMM and NAM can take place, as well as the potential degradation of DMF, which produces dimethylamine, an impurity basic enough to initiate polymerization *via* AMM. The latter impurity might also explain the presence of slight auto polymerization observed at the end of the kinetic screening in the NCA monomer syringe (5-13%, **Figure S6**). Regardless, flow and batch-synthesized polypeptides bear high resemblance to each other. Translation of NCA ROP to a flow process highly accelerates the synthesis and provides a scalable process with high potential for future applications, such as scaled commercial synthesis of polypeptides, and replacement of single-use plastics with completely biodegradable materials using polypeptide polymers.

With the very significant rate increase in flow, we further tested our hypothesis outlined above. Therefore, we compared the flow reaction with an identical process using gas impermeable tubing (i.e. gastight perfluoroalkoxy tubing). As shown in **Table 1** (entry 1) this condition led to a much lower polymerization rate compared with gas permeable tubing (**Table 1**, entry 3). Additionally, the

conversion *vs.* time trend is similar to batchwise NCA ROP as shown in the supporting information in **Figure S7** (green diamonds *vs* blue triangles). Polypeptides synthesized in this work in batch were synthesized in a closed system, hence observation of similar trends in batch and gas impermeable tubing would be logical. Next, we applied different external conditions to the tubing for reactions performed using ZLL NCA in gas permeable silicon (*i.e.* normal pressure, vacuum and CO_2 environment). Comparing a highly saturated CO_2 environment with a *normal* air environment for a reaction with a residence time of 20 minutes clearly demonstrated vast reduction in polymerization rate (from 72% to 21% monomer conversion for respectively air and CO_2 environments, **Table 1** entry 2 *vs.* 4). This observation corroborates our hypothesis that gas permeable tubing accelerates NCA ROP due to efficient removal of CO_2. Evacuated conditions were also investigated to examine whether a surrounding vacuum further accelerates the process; however, we only observed a slight increase in conversion (from 72% to 75%, slight increase in M_n, **Table 1** entry 2 *vs* 5 and **Figure S8**).

Given that atmospheric conditions already vastly accelerate the NCA ROP we chose to further use this convenient low-cost flow system in a following reaction in which a lower amount of initiator was used to reach a higher DP (from 0.025 M to 0.005 M, **Table 1**, entry 6 and entry 8). This was performed for ZLL and BLG NCAs. Interestingly this gave a slightly different conversion *vs*. time profile (**Figure S7**). Limited polymerization was observed in the first 40 minutes (around 10%), followed by a faster rate similar to the former condition (i.e. DP_{target} = 20, $[ZLL\ NCA]_o$ = 0.5M). This apparent "inhibition period" has not been observed in literature for this type of NCA ROP in DMF and is under our careful investigation. We suggest this is potentially caused by low initiator concentrations and hence higher sensitivities to impurities. Regardless, the polymerization still proceeds well, and the synthesis of higher DP (DP_{target} = 100) poly(lysine) could be realized. Molecular weight measurements *via* SEC of these higher DP polypeptides can be found in the supporting information (**Figure S9**, M_n = 8700 g · mol^{-1} at 80 minutes residence time). The measured M_n of all polypeptides synthesized in this work deviates from the theoretical molecular weights, and is most likely due to the fact that a calibration against PMMA standards was used to derive the

molecular weight. The hydrodynamic volume relation of a polypeptide is significantly different from a polymethacrylate.[43] Interesting to note here as well is the slightly reduced bimodality compared with the lower DP conditions (DP_{target} = 20).[43] BLG appears to react slower compared with ZLL in the conversion *vs* time profile (see **Figure S7**, empty red triangles) reaching a monomer conversion of 47% after 80 minutes residence time. Nevertheless, a monomodal distribution with M_n = 11700 and $Đ$ = 1.27 is achieved under this condition (see **Figure S10 B** and **Table 1** entry 8). This demonstrates that defined polypeptides can be achieved with this flow setup.

To further investigate the wide applicability of the flow reactor design, we also investigated the synthesis of polypeptide stars. This was achieved through the use of triethylaminetriamine (TREN) as initiator for the ring opening polymerization of BLG NCA, which should furnish 3-arm star polypeptides. TREN is a privileged initiator, given that the combination of three primary amines and the core tertiary amine offers a cooperative effect which results in rapid polymerization kinetics.[50] To our satisfaction, employment of TREN in our flow process also yielded enhanced kinetics (**Figure S7**, orange circles) for $DP_{target\ per\ arm}$ = 20, $[BLG]_0$ = 0.5 M. Within 10 min, 50 % consumption of NCA was apparent, and by 30 min the polymerization had reached full conversion. Analysis of the resultant polypeptide by SEC revealed polypeptides with M_n = 3200 g·mol^{-1} and $Đ$ = 1.49 (See **Table 1** entry 9 and **Figure S12**). The molecular weight is lower than expected, but this can be explained by the fact that star polymers have a different hydrodynamic volume relation than linear polymers (by which SEC calibration was performed) of the same molecular weight, due to their more compact nature.[51] The outcome of this experiment suggests that other NCA ROP systems with enhanced kinetics may be successfully translated to continuous flow as well.

The flow rates that were used in this work together with the dimensions of the flow reactor ensure a laminar flow regime. In such a flow regime the mixing of all reagents in the radial direction is limited by diffusion. It should be noted that observations on polymerizations performed in such a reactor design demonstrated that different flow rates can lead to broadening of the residence time

distribution. This broadening is then further correlated with an increase in *Đ* and a decrease in both M_n and conversion as shown by Leibfarth and coworkers.[52] Optimization on this part could be achieved for example by turbulent flow conditions, which is unfortunately not feasible in our lab scale setup, but could nonetheless be conveniently addressed in further upscaling. Regardless, the presented flow setup provides an alternative option for accelerated NCA ROP in a convenient solvent with high polarity in which many functional NCAs are soluble.[53]

A first upscale of the reaction to a significant production is already realized with our present setup. In future upscaling we hypothesize that a tube-in-tube design would be most effective to ensure optimal removal of CO_2 along the length of the flow reactor, similar to the setup described by Ley and coworkers.[54] Alternatively, the use of reactor parallelization and/or the use of longer reactor tubing to enable a larger output with very small batch-to-batch variation is directly possible without too much difference in the design.[18, 55] Other polymerization methods (e.g. reversible deactivation radical polymerization and anionic polymerization) have already strongly benefited from continuous flow chemistry; we therefore hope to stimulate more implementation of continuous flow strategies in the field of NCA ROP as well.

In conclusion we developed a facile continuous flow approach for polypeptide production *via* the NAM. More efficient removal of CO_2 in flow was afforded by gas permeable silicon tubing as polymerization reactor. Employing flow chemistry was the key factor of success given the much larger surface area of the flow tubing compared with classical batch reactors. This translated to an accelerated system reaching 92% conversion within 40 minutes compared to 6 hours when performed in batch (9.5-fold increase). The continuous flow reactions were repeated with BLG NCA as monomer of which a similar increase in reaction was observed, but which allowed a more straightforward molecular weight analysis. Formation of higher DP or various (block)copolypeptides *via* flow reactor cascade design or the synthesis of complex polypeptide structures in flow is a valuable avenue to explore next. This work provides a proof of concept for future development on

NCA ROP performed in flow towards streamlining the manufacturing of relevant and useful polypeptide materials suited for a wide variety of applications, from biomedical fields to fundamental material science.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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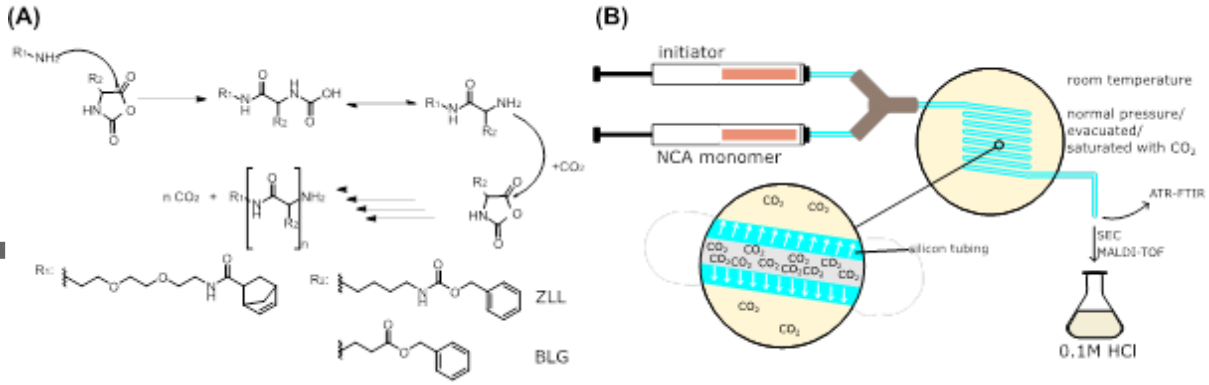


Scheme 1. (A) N-carboxyanhydride (NCA) ring opening polymerization (ROP) *via* the normal amine mechanism (NAM) of Nε-Benzoxycarbonyl L-Lysine (ZLL) or γ-benzyl-L-glutamate (BLG) NCA with norbornene initiator containing primary amine functionality (NB-NH2). (B) Schematic description of the flow setup in the current work. Various external influences can be applied such as normal pressure, vacuum or CO_2 saturation.

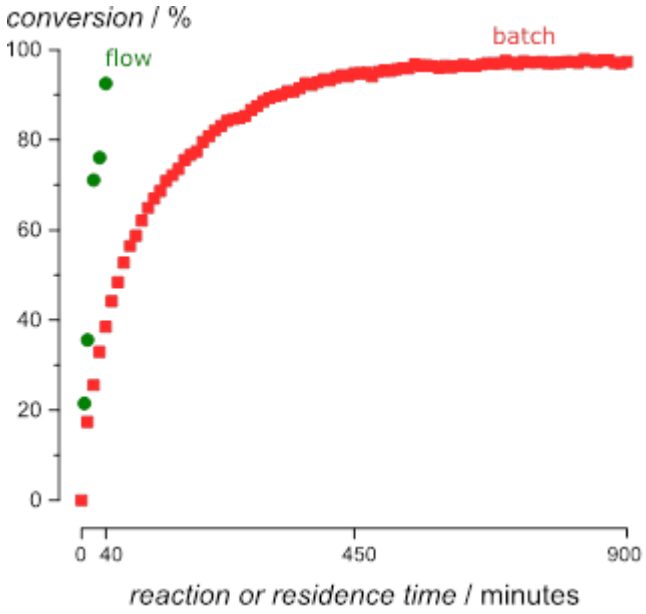
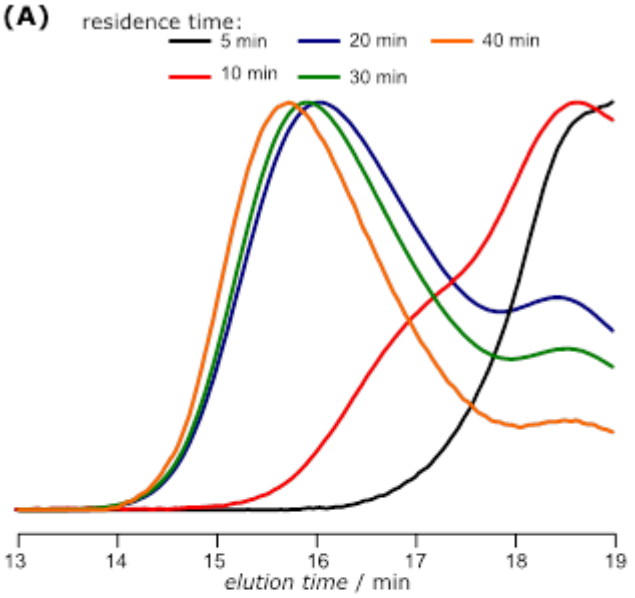
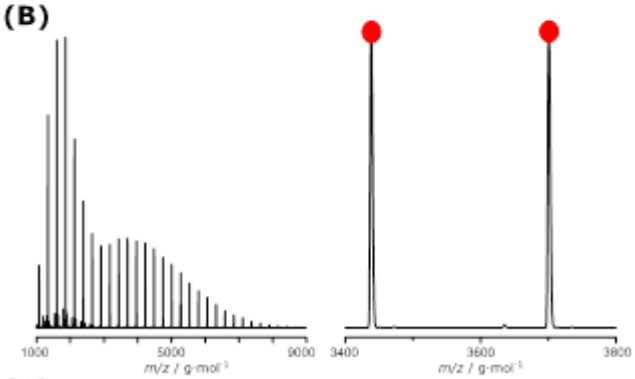


Figure 1. NCA monomer conversion over time for ROP of ZLL by NB-NH_2 in batch (red squares) and flow with gas permeable silicon tubing (green circles). DP_{target} = 20, $[NCA]_0$ = 0.5M. Conversion was monitored *via* ATR-FTIR by following the decrease in absorbance at 1850 cm^{-1}(C=O stretch).





(C)

α group	ω group	Nos. NCA	ion	$m/z_{exp.}$	m/z_{theo}
Norbornene	NH_2	11	Na^+	3438.48	3439.05
Norbornene	NH_2	12	Na^+	3700.71	3701.36

Figure 2. SEC traces (A) of poly(lysine) collected and quenched at different kinetic timepoints in flow using gas permeable silicon tubing. DP_{target} = 20, $[ZLL]_0$ = 0.5M. MALDI-TOF analysis of polypeptides collected and quenched at 20 minutes residence time (B) with assignment of observed peaks (C).

Table 1. Summary of performed NCA ROP experiments in flow.

entry	tubing type	surrounding condition	DP_{target}	[I] (M)	$[NCA]_0$ (M)	residence time (min)	monomer conversion	M_n ($g \cdot mol^{-1}$)	$Đ$[a]
1	gastight	air	20	0.025	0.5 (ZLL)	40	35%	-	-
2	gas permeable	air	20	0.025	0.5 (ZLL)	20	72%	3900[a]	1.79
3	gas permeable	air	20	0.025	0.5 (ZLL)	40	92%	5200[a]	1.69
4	gas permeable	CO_2	20	0.025	0.5 (ZLL)	20	21%	-	-
5	gas permeable	vacuum	20	0.025	0.5 (ZLL)	20	75%	4800[a]	1.91
6	gas permeable	air	100	0.005	0.5 (ZLL)	80	75%	8700[a]	1.51
7	gas permeable	air	20	0.025	0.5 (BLG)	40	92%	5600[b]	1.40[b]
8	gas permeable	air	100	0.005	0.5 (BLG	80	47%	11700[b]	1.27[b]
9	gas permeable	air	20[c]	0.008	0.5 (BLG)	30	>99%	3200[a]	1.49[a]

[a]Measured in DMF and calibrated against narrow PMMA standards. [b]measured in dimethylacetamide and calibrated against narrow PMMA standards. [c]DP targeted per arm of the star for NCA ROP performed with TREN

N-carboxyanhydride ring opening polymerization in traditional systems often suffers from slow polymerization. Substantial acceleration was observed in this work by performing the polymerization in a silicon microflow reactor due to more efficient removal of the CO_2 byproduct. This continuous flow proof of concept can further stimulate the use of continuous flow for polypeptide synthesis potentially streamlining N-carboxyanhydride ring opening polymerization.

_N_-carboxyanhydride ring opening polymerization in continuous flow

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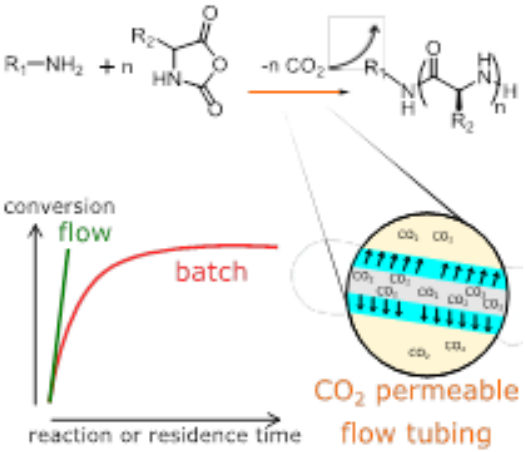
Accelerated Polypeptide Synthesis *via* N-carboxyanhydride Ring Opening Polymerization in Continuous Flow



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