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Simultaneous Polymerization and Polypeptide Particle Production via Reactive Spray-Drying

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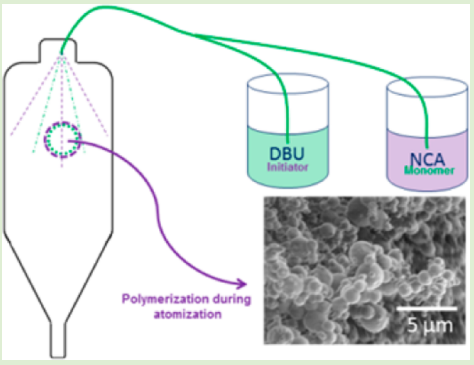
Supporting Information

ABSTRACT: A method for producing polypeptide particles via *in situ* polymerization of *N*-carboxyanhydrides during spray-drying has been developed. This method was enabled by the development of a fast and robust synthetic pathway to polypeptides using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as an initiator for the ring-opening polymerization of *N*-carboxyanhydrides. The polymerizations finished within 5 s and proved to be very tolerant toward impurities such as amino acid salts and water. The formed particles were prepared by mixing the monomer, *N*-carboxyanhydride of L-glutamic acid benzyl ester (NCA_{Glu}) and the initiator (DBU) during the atomization process in the spray-dryer and were spherical with a size of ~1 μm. This method combines two steps; making it a straightforward process that facilitates the production of polypeptide particles. Hence, it furthers the use of spray-drying and polypeptide particles in the pharmaceutical industry.



INTRODUCTION

The concept of small particles that are able to deliver active compounds to specific locations in the body is one of the central aims in the pharmaceutical industry, and a lot of the current research is based on the development of new drug/particle formulations. The particles have to stabilize and solubilize the active compound in the dispersion media in order to facilitate delivery to the desired site as well as to obtain a slow release.[1,2] These particles can take different shapes, such as self-assembled micelles and solid polymer particles.[3,4]

One of the more elegant methods of preparing these drug/particle formulations is spray-drying.[5,6] This is a one-step process that uses mild conditions and is hence very suitable for sensitive samples such as proteins. It also has high reproducibility, efficacy and better control over particle morphology and shape compared to many other preparation methods.[7−9] The spray-drying method also has a low energy-consumption and scaling-up possibilities making it a very desirable method for use in the industry.[9,10] It has for these reasons been applied in the pharmaceutical industry for a long time, yet there is a lot more to uncover.

There are many different polymers that have been spray-dried, varying for natural polymers such as chitosan[11] to synthetically prepared polylactide (PLA)-based materials.[12,13] For biomedical application, most of the research revolves around these.[14−16] Polypeptides are another group of polymers that is interesting for drug delivery applications due to their biocompatibility, versatility, and their compatibility with protein and peptide drugs.[17−19] There are some few examples in literature of spray-drying of natural polypeptides (extracted from plants),[20] but more interestingly, synthetic polypeptides have not been used for preparation of particles via spray-drying. One advantage of using synthetic polypeptides compared to natural is the ability to tailor polypeptides with desired properties. These polypeptides can be obtained by, e.g., ring-opening polymerization (ROP) of *N*-carboxyanhydrides (NCAs) using amines as initiators.[19,21,22] Such polymerizations have proved to be highly sensitive and need vigorous purification of the monomers (NCAs), as impurities within can cause side-reactions such as termination.[23,24] In addition to the impurities present within the monomers, the polymerizations are sensitive toward moisture and need to proceed under an anhydrous and inert atmosphere. Primary amines are often the first choice as initiators due to their abundancy and the facile synthetic procedures needed to convert a variety of functional groups to amines, which then can be used as initiators.[25−27] Kricheldorf et al. among others have also reported that tertiary amines (DMAP, pyridine etc.) also could initiate polymerization of NCAs by the induction of zwitterions.[28] However, these polymerizations have very long polymerization times, spanning over several days. More recently, even 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) has been used to initiate zwitterionic polymerization of *N*-butyl-*N*-carboxyanhydrides (Bu-NCA).[29]

The preparation of polymer particles usually requires several steps, e.g., synthesis, purification and then spray-drying, making it a tedious process. A move toward reducing the amount of steps has been taken in our group, where cross-linking of

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hemicellulose was conducted during spray-drying.[30] Our aim here is to take this concept one step further by combining polymerization and spray-drying into one step in order to make it more efficient, use less solvent and obtain a higher yield when going from monomer to particles. To be able to combine these we need a polymerization method that is robust, tolerating moisture and other impurities. In addition to this, the polymerization needs to be fast as it will need to occur during the atomization process in the spray-dryer. We hypothesize that by using amidine or guanidine bases as initiators in the ring-opening polymerization of *N*-carboxyanhydrides, we will obtain a fast polymerization. As an effect of this, any impurities or moisture that is present within monomer, solvent or surrounding environment will have no time to interfere with the polymerization making this route robust. By being able to conduct the polymerization of polypeptides *in situ* in the spray-dryer, we can go directly from monomer to polymer particle without the intermediate step of polymerization, making this a straightforward and more viable method for preparing polypeptide particles for pharmaceutical applications.

EXPERIMENTAL SECTION

Materials. Ethyl acetate (anhydrous, 99.8%), triphosgene (98%), (+)-α-pinene, 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD), 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (mTBD), 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU), S-benzyl-L-cysteine (97%, Cys), L-glutamic acid γ-benzyl ester (99%, Glu), diethyl ether (99.8%), hexylamine (98%), L-phenylalanine (PhAla, 98%) dimethylformamide (DMF, anhydrous, 99.8%), dichloromethane (DCM, anhydrous, 99.8%) and dithranol (MALDI matrix) were all received from Sigma-Aldrich. Heptane (GPR Rectapur), tetrahydrofuran (THF, LiChrosolv), and acetic acid (AcOH, 100%) were received from VWR. Chloroform (99%) was received from Fisher Scientific, and all chemicals were used without further purification.

Synthesis of N-Carboxyanhydrides (NCAs). *N*-Carboxyanhydrides of L-glutamic acid γ-benzyl ester, phenyl alanine and S-benzyl-L-cysteine were prepared according to the literature with some modifications.[31] Briefly, after drying under reduced pressure, the derived amino acid was suspended in dry ethyl acetate under a N_2-atmosphere. α-Pinene was added to the suspension and worked as a HCl scavenger. Next, triphosgene was dissolved separately in dry acetate and added slowly to the suspension. Within 1 h, the suspension turned to a homogeneous solution, and the reaction proceeded for 3 h at 80 °C. After the reaction, the homogeneous solution was precipitated in cold heptane and filtrated. The NCAs were purified by three sequential recrystallizations from a 2:1 heptane/ethyl acetate mixture and then dried under reduced pressure with the exception of the NCA_{Glu} used for the elucidation of the impact of the impurities on the polymerization which was only purified by one precipitation in heptane and dried under reduced pressure.

Elucidation of NCA Polymerization Parameters. Polymerization of the NCA_{Glu} was conducted using the guanidine or amidine bases; TBD, mTBD, and DBU as initiators; and DMF, DCM, or acetone as solvent. Generally, the monomer and initiator were weighed separately in flame-dried Schlenk flasks and dissolved either in dry DMF, DCM, or THF (0.1 M NCA and 0.13 M initiator (stock-solution)). The monomer and initiators were stored under an inert atmosphere. Once the NCA was completely dissolved, the initiator (0.1 mL of stock solution for $M_{n,theo}$ = 15 000 g/mol) was added quickly, and the reactions were allowed to proceed for 10 min under a N_2-flow. Reaction samples were withdrawn at 5 s, 20 s, 40 s, 60 s, 3 min and 5 min. The reaction was terminated by precipitated in cold diethyl ether and dried under reduced pressure. In some cases the polymerization was first terminated by the addition of acetic acid in order to ensure quenching of the initiator. [M]/[I] was varied to obtain theoretical molecular weights of 5000, 15 000, and 25 000 g/mol. The copolymers were prepared according to a similar procedure as for the homopolymers. Briefly, both NCAs were dissolved together in dry DMF and 0.1 mL DBU (stock-solution) was added. Purification was performed in the same manner as for the homopolymers.

Reactions purely in solvent (no initiator added), DMF or DCM, were performed for NCA_{Glu} and were allowed to proceed for 24 h. The solvent was then removed under reduced pressure.

Polymerizations of NCA_{Glu} were also performed without N_2-purging or dried and purified solvents as well as with the addition of water to determine the tolerability of the polymerization method. The polymerizations were prepared in a similar manner as above. The DBU was kept under an inert atmosphere prior to use.

In Situ Polymerization and Particle Preparation. The particles were prepared by a spray atomization technique, spray-drying, using a mini spray-dryer (B-290, Büchi) equipped with an inert loop (B-295, Büchi). A 0.7 mm two-fluid nozzle was used and the inlet temperature was set to ~10 °C above boiling point of the selected solvent ($CHCl_3$ − ~76 ± 3 °C and THF − 80 ± 3 °C). The aspirator worked at 100% meaning a gas flow of 35 m^3/h while the N_2-flow was kept at ~670 L/h. Two different concentrations were used for the spray-drying, 2.5 and 5 mg/mL. For the *in situ* polymerization, one tube from the monomer solution (2.5 mg/mL, 500 mL) and second from the initiator solution (0.15 mg/mL, 500 mL) were joint just before entering the nozzle to ensure polymerization occurring at the appropriate time point. After the particles were collected, the yield was calculated and the samples were stored in a desiccator until further characterization.

Characterization. The structures of the monomers and their respective polymers were characterized by ^{1}H NMR and ^{13}C NMR. A Bruker Avance 400 Hz NMR was used for the structure determination. All samples were analyzed in $CDCl_3$, and the residual solvent peak was used as the reference (δ = 7.26 ppm or δ = 77.16 ppm).

The molecular weight (M_n) and dispersity ($Đ$) of the synthesized polypeptides were determined by size exclusion chromatography using a TOSOH EcoSEC HLC-8320 GPC system equipped with 3 columns (PSS PFG 5 μm; Microguard, 100 and 300 Å) from PSS GmbH and an EcoSEC RI detector. The eluent was 0.01 M LiBr in DMF (0.2 mL/min), and the measurements were performed at 50 °C. Broad and linear poly(methyl methacrylate) standards were used for the calibration, and toluene was used as the internal standard.

The morphology of the particles was determined by scanning electron microscopy (SEM) using a Hitachi S-4800 SEM. The samples were sputter-coated with Ag/Pd (Cressington 208HR sputter-coater). An accelerating voltage of 0.7−1.0 kV was used. The size of the particles is reported as the mean diameter of 10 particles.

RESULTS AND DISCUSSION

Polymer particles can be used as drug delivery vehicles for, e.g., proteins and peptides; in order to obtain good compatibility polypeptides can be used. Commonly, the preparation of polymer particles demands at least three steps, synthesis of polymer, purification, and preparation of the particles and hence requires much solvent and long production times. We therefore developed an effective pathway for the preparation of polypeptide particles directly from *N*-carboxyanhydrides, in one step. The *in situ* polymerization of polypeptides during spray-drying using amidine or guanidine bases eliminates additional steps and consecutively makes this a straightforward and more viable process for the industry.

Elucidation of *N*-Carboxyanhydride Polymerizations Parameters. To enable the in situ polymerization during spray-drying, the development of a robust polymerization method of NCAs was needed. The monomer, *N*-carboxyanhydride of L-glutamic acid γ-benzyl ester (NCA_{Glu}) was successfully prepared using conventional synthetic procedures, Supporting Information (S1−S3).[31,32] Initial polymerizations of NCA_{Glu} (without spray-drying) were performed by a fast addition of the DBU solution (initiator in DMF) to the

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monomer in DMF and the reaction proceeded under inert atmosphere at ambient temperature for 10 min. The polymerizations were successful and were characterized by NMR and SEC.

The NMR analysis showed that the peak at $\delta \sim 6.3$ ppm, corresponding to −NH− in the monomer, disappeared as a new peak at $\delta \sim 8.4$ ppm appeared, corresponding to the amide in the polymer backbone and hence confirming ring-opening of the monomer, Figure 1. The SEC traces exhibited a monomodal peak, with a molecular weight of 19 600 g/mol (sample 1) and a dispersity of 1.5, Table 1. The observed overestimation of the molecular weights can be due to the difference in hydrodynamic volume between the polypeptide and the standards used for calibration or due to the formation of secondary structures.[33]

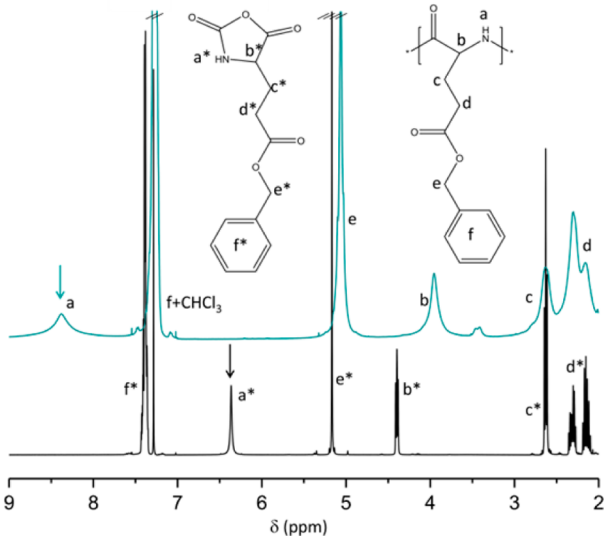

Figure 1. ^{1}H NMR spectra of NCA_{Glu} (bottom) and PGlu (sample 1, top).

The [M]/[I] ratio was altered and polypeptides with three different theoretical molecular weights were prepared. The molecular weight exhibited a clear dependence on [M]/[I], with an increase in molecular weight occurring with increased [M]/[I] (Table 1). However, no prediction of the molecular weight could be obtained, as has previously been observed for the DBU-initiated polymerization of lactide.[34] The molecular weights varied from 19 600−90 700 g/mol with increased [M]/[I] and had dispersities ranging from 1.5 to 1.8.

Aliquots were withdrawn during the first 5 min of the polymerization and analyzed with ^{1}H NMR and SEC. The aliquotes were quenched by the addition of acetic acid in order to ensure deactivation of the initiator. The ^{1}H NMR spectra indicated that all monomer was consumed within 5 s of the addition of DBU, as the peak for −NH− corresponding to the monomer (~6.3 ppm) disappeared and was replaced by the amide peak of the polymer (~8.4 ppm) (Figure 2). As the polymerization proceeds, the spectra remained identical, indicating the completion of the polymerization within only 5 s.

The short polymerization time was supported by the SEC results, as the final molecular weight was reached after only 5 s, and no change in molecular weight was observed during the remaining reaction time (Figure 2).

To expand the use of the bicyclic base initiated NCA polymerizations, the guanidine bases TBD and mTBD (samples 5 and 6) were evaluated as initiators. Both TBD and mTBD could initiate the ROP of NCA. The polymerization using TBD proved to lack control, yielding different results under identical reaction conditions while the polymerizations using mTBD exhibited similar trends in molecular weight and dispersity as DBU, Table 2. The NCA_{Glu} polymerizations were conducted in DCM as well (sample 7−9) and gave rise to polymers with very high molecular weights as measured by SEC, Table 2.

Solvent-induced polymerizations have previously been reported[35] and therefore, polymerizations in DMF and DCM were evaluated without the presence of an initiator. The polymerizations were conducted in the same manner as for the DBU-initiated polymerizations but with a polymerization time of 24 h and without the addition of an initiator. The SEC results showed small traces of oligopeptides after 24 h, but most of the monomer remained unreacted (Supporting Information, Figure S4). Solvent-induced polymerization within 10 min was hence disregarded.

Copolymers of NCA_{Glu} with either NCA_{PhAla} or NCA_{Cys} were prepared in DMF, using DBU as initiator in 1:1 and 2:1 ratio (NCA_{Glu}:$NCA_{Cys/PhAla}$, sample 11−14). The presence of peaks corresponding to both PGlu and PCys or PPhAla in the ^{1}H and ^{13}C NMR spectra indicate successful copolymerization, and the absence of the −NH− peaks corresponding to the NCAs indicate quantitative conversion suggesting the possibility of polymerizing a wider array of α-amino acid NCAs using DBU (Table 2 and Supporting Information Figure S5). Homopolymers of NCA_{PhAla} and NCA_{Cys} had poor solubility common organic solvents and could hence not be analyzed.[36,37]

The rates of the DBU-initiated polymerizations (completion within 5 s) suggest that these polymerizations could be quite robust and that impurities within the monomer or moisture would have a small influence on the polymerizations. The effect of an inert atmosphere on the polymerization procedure was explored by conducting the polymerization of NCA_{Glu} in an NMR-tube using $CDCl_3$ (not purified) as the solvent and in air. The disappearance of the monomer peak at $\delta \sim 6.3$ ppm (−NH−) in the ^{1}H NMR spectrum and the appearance of the polymer peak at $\delta \sim 8.4$ ppm indicate that polymerization occurred, Figure 3.

The obtained polymer had a molecular weight of 6800 g/mol and a dispersity of 2.0. A similar polymerization (sample 15) was also conducted in DMF (not purified or dried), and resulted in a polypeptide with similar characteristics as the

Table 1. Polymerization Conditions and Characterization of the Prepared Polypeptides

sample	monomer	initiator	solvent	time	$M_{n,\,theo}$ [g/mol]	$M_{n,\,SEC}$[g/mol]	$Đ_{SEC}$
1	NCA_{Glu}	DBU	DMF	10 min	5000	19 600	1.5
2	NCA_{Glu}	DBU	DMF	10 min	15 000	24 300	1.5
3	NCA_{Glu}	DBU	DMF	10 min	25 000	90 700	1.8
4	NCA_{Glu}	hexylamine	DMF	48 h	5000	7800	1.2

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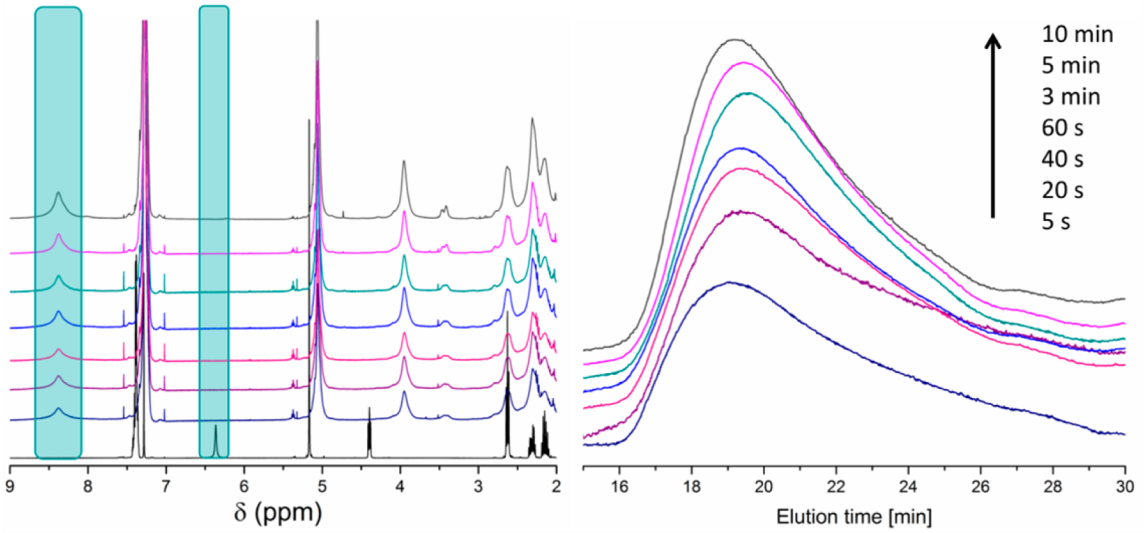

Figure 2. ^{1}H NMR (right) and SEC traces (left) of PGlu (sample 1) at different reaction times.

Table 2. Polymerization Conditions and Characterization of the Prepared Polypeptides Using Different Monomers, Initiators and Solvents[a]

sample	monomer	initiator	solvent	$M_{n,theo}$ [g/mol]	$M_{n,SEC}$ [g/mol]	$Đ_{SEC}$
2	NCA_{Glu}	DBU	DMF	15 000	24 300	1.5
5	NCA_{Glu}	TBD	DMF	15 000	6400	2.8
6	NCA_{Glu}	mTBD	DMF	15 000	38 400	1.5
7	NCA_{Glu}	DBU	DCM	15 000	360 700	2.0
8	NCA_{Glu}	TBD	DCM	15 000	232 800	2.0
9	NCA_{Glu}	mTBD	DCM	15 000	275 400	2.6
10	NCA_{Glu}/ NCA_{Cys}	DBU	DMF	5000 (1:1)	17 200	1.6
11	NCA_{Glu}/ NCA_{Cys}	DBU	DMF	15 000 (2:1)	56 200	1.5
12	NCA_{Glu}/ NCA_{PhAla}	DBU	DMF	5000 (1:1)	10 500	1.8
13	NCA_{Glu}/ NCA_{PhAla}	DBU	DMF	15 000 (2:1)	12 500	1.7

[a]The polymerization time is 10 min.

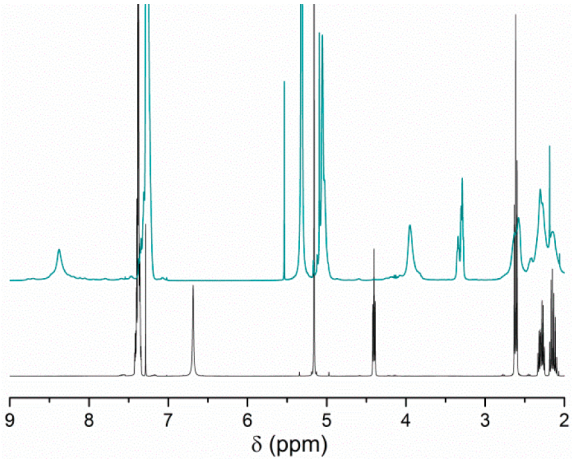

Figure 3. ^{1}H NMR spectra of the polymerization of NCA_{Glu} in a NMR tube.

polymerization conducted in dry and purified DMF under an inert atmosphere (Table 3).

Water is another impurity that might cause side reactions during NCA polymerization. The tolerance of the polymerization toward water was explored by the addition of water (a few drops) to a DMF solution of the monomer, after this DBU was added (sample 17). The ^{1}H NMR and SEC indicated a successful polymerization indicating the DBU initiated polymerization of NCA is not sensitive toward the presence of water within the reaction mixture (Table 3).

The development of the amidine- or guanidine-initiated polymerization of NCA gives rise to a robust and fast polymerization method that is suitable to perform *in situ* during spray-drying.

Spray-Drying of Polypeptide Particles. Spray-drying is an effective and straightforward method for obtaining particles, commonly applied in the industry.[6] As mentioned above, the developed polymerization method was very robust and was tested *in situ* during the atomization process in the spray-drier. The solvent chosen for the *in situ* polymerization were THF and $CHCl_3$. THF was chosen as a substitute for DMF due to the high boiling point of DMF (faster evaporation gives a higher production yield[38]), as it is a polar, aprotic solvent while $CHCl_3$ was chosen as a substitute for DCM, since it has a slightly higher vapor pressure (too fast evaporation gives rise to

Table 3. Characterization of the Polypeptides Obtained under Robust Polymerizations Conditions

sample	monomer	initiator	solvent	time	$M_{n,theo}$ [g/mol]	$M_{n,SEC}$ [g/mol]	$Đ_{SEC}$
14[a]	NCA_{Glu}	DBU	DMF	10 min	15 000	27 600	1.7
15[b]	NCA_{Glu}	DBU	DMF	10 min	15 000	32 000	1.7
16[c]	NCA_{Glu}	DBU	DMF/H_2O	10 min	15 000	33 000	1.5

[a]Not dried or purified, polymerization performed in air. [b]Monomer not extensively purified, only through 1 precipitation. [c]A few drops of water were added to the monomer solution in DMF.

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collapsed particles[9]). Both the chosen solvents behaved similarly during polymerization as the solvents they were replacing. The *in situ* polymerization and particle preparation was conducted by separately dissolving the monomer (NCA_{Glu}) and initiator (DBU) in $CHCl_3$ or THF and later mixing the solutions together in the tubing just before reaching the nozzle (atomization). Two monomer feed concentrations (2.5 and 5 mg/mL) were evaluated in order to obtain the a better spray-drying process; the inlet temperature was kept approximately 10 °C above the boiling point of the solvent in order to ensure fast drying. Spherical particles were obtained for both monomer feed concentrations using $CHCl_3$ (Figure 4). However, using

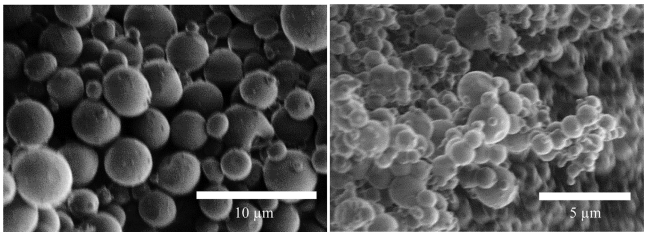

Figure 4. (a) Particles formed from a monomer feed concentration of 5 mg/mL and (b) from a monomer feed concentration of 2.5 mg/mL using $CHCl_3$ as the solvent.

THF as solvent gave a low yield of particles at a monomer feed concentration of 5 mg/mL since most of the polymer formed a film on the cyclone wall while the lower concentration (2.5 mg/mL) only gave rise to a film (Supporting Information, Figure S6). THF was, hence, eliminated as a solvent for the *in situ* polymerization. The spray-drying conditions that worked best were using $CHCl_3$ as solvent and a concentration of 2.5 mg/mL.

The size of the particles was dependent on the monomer concentration where the lower concentration gave rise to smaller particles with smooth surfaces while the higher concentration gave rise to larger particles with slightly more rough surfaces. The occurrence of the *in situ* polymerization was confirmed by ^{1}H NMR and SEC. The ^{1}H NMR spectra show that the peak corresponding to the −NH− in the monomer ($\delta \sim 6.3$) was replaced by a peak at $\delta \sim 8.4$, indicating ring-opening of the monomer and hence polymerization, Figure 5. The successful polymerization was further confirmed by SEC, which indicated molecular weights above 6000 g mol^{-1}. All conditions gave rise to a polymerization independent of solvent and monomer feed concentration.

While the choice of solvent did not affect the size and shape of the particles (~4 μm, spherical), it had an effect on the *in situ* polymerization. Just as for the polymerization conducted under regular conditions, using a polar and aprotic solvent such as THF or DMF yielded higher control compared to the nonpolar $CHCl_3$ and DCM. This was indicated by the lower dispersity obtained when using THF compared to $CHCl_3$ (2.19 and 5.17), Table 4.

The morphology and shape of spray-dried particles is dependent on the intrinsic viscosity of the solution and hence on the molecular weight of the polymer. However, the obtained particles showed no dependence on the molecular weight. This is because the polymerization occurs during and after the critical point (atomization) and therefore all solutions have the same intrinsic viscosity (dependent on monomer concentration) prior to atomization. This means that the molecular weight of the polypeptide constituting the particles can be altered without having to change the parameters for the spray-drying process.

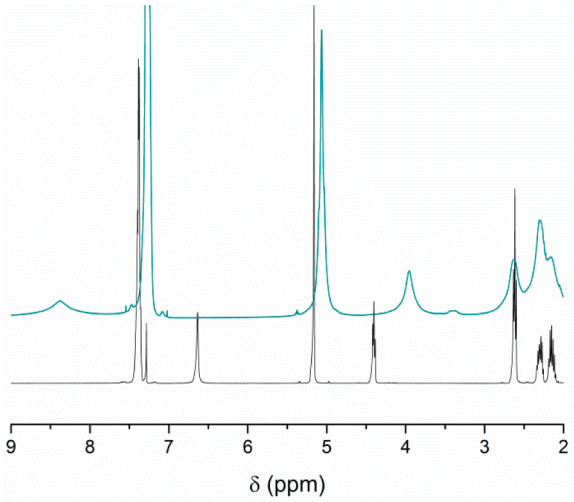

Figure 5. ^{1}H NMR spectra of NCA_{Glu} (bottom) and particles of PGlu after *in situ* polymerization during spray-drying ($CHCl_3$, monomer feed 2.5 mg/mL).

Table 4. Characterization of the Prepared Particles via Spray-Drying Using *in Situ* Polymerization

sample[a]	solvent	conc. [mg/mL]	M_n/Đ [g/mol]	morphology[b]
NCA_{Glu}/DBU	$CHCl_3$	5	14 300/5.17	spherical, ~3 μm
NCA_{Glu}/DBU	$CHCl_3$	2.5	6 800/5.44	Spherical, ~1 μm
NCA_{Glu}/DBU	THF	5	10 000/2.19	Spherical, ~4 μm
NCA_{Glu}/DBU	THF	2.5		-[c]

[a]Gas flow 55, aspirator 100%, pump 30% during all polymerizations. [b]An average size determined by SEM. [c]Only film was formed.

The monomer was also spray-dried without the initiator, and the particles obtained were spherical (M_n = 170 g/mol) and similar size as the DBU-initiated polypeptide particles, Table 5.

Table 5. Characterization of the prepared particles via spray-drying of PGlu, NCAGlu, and *in Situ* Polymerization of *S*-Benzyl-Cysteine

sample[a]	conc. [mg/mL]	M_n/Đ [g/mol]	morphology
NCA_{Glu}	4.8	170/1.1	spherical, ~2 μm
P_{Glu}	2.5	19 900/1.5	collapsed,
P_{Glu}	4.8	22 000/1.6	collapsed, 1−10 μm
NCA_{Cys}/DBU	2.5	-[b]	spherical, < 1 μm

[a]Gas flow 55, aspirator 100%, pump 30% during all polymerizations. [b]Not soluble in common organic solvents.

However, the particle yield was low due film formation on the cyclone walls (similar for THF as solvent), Supporting Information (Figure S7). Using polymer particles instead of monomer particles will enable better stability (difference in solubility) as well as the possibility of surface functionalization such as deprotection of the carboxyl group of L-glutamic acid γ-benzyl ester making it hydrophilic but without making the particles completely soluble in water.

Another advantage of conducting the polymerization *in situ* during the spray-drying is that particles can be prepared from polypeptides with poor solubility in organic solvents such as

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poly(*S*-benzyl-cysteine). The particles made of poly(*S*-benzyl-cysteine) were prepared in the same manner as the particles made out of PGlu; *S*-benzyl-cysteine and DBU were dissolved in $CHCl_3$ separately at a concentration of 2.5 mg/mL and spray-dried under the same conditions (Figure 6). The particles

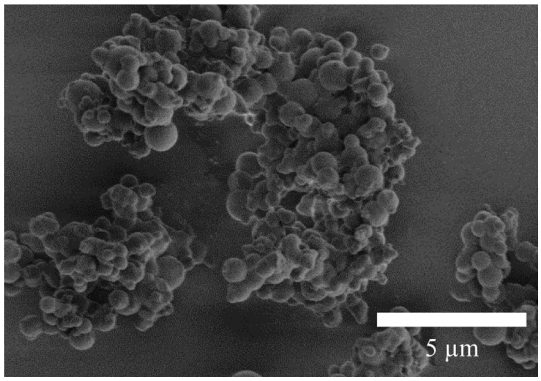

Figure 6. Particles formed from a monomer (NCA_{Cys}) feed concentration of 2.5 mg/mL using $CHCl_3$ as the solvent.

were spherical with sizes <1 μm and could not be dissolved in $CHCl_3$ indicating that polymerization had occurred (monomer is fully soluble in $CHCl_3$), Table 5. This indicates that spray-drying with *in situ* polymerization can be used for different NCAs and hence copolymerization is possible as well.

Spray-drying of already prepared PGlu in $CHCl_3$ was also performed. The particles formed were collapsed at both 2.5 and 5 mg/mL (Figure 7, Table 5 and Supporting Information

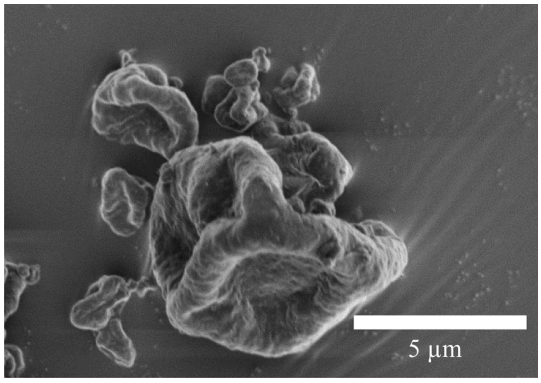

Figure 7. Morphology of spray-dried PGlu in $CHCl_3$ (monomer feed concentration 5 mg/mL).

Figure S8). The collapsed particles are a consequence of PGlu not being fully soluble in $CHCl_3$.[9] This confirms the advantage of performing *in situ* polymerization.

CONCLUSIONS

In situ polymerization of *N*-carboxyanhydrides during spray-drying was successfully applied to obtain polypeptide particles. To be able to perform the *in situ* polymerization, we developed a fast and highly robust pathway for synthesizing polypeptides using amidine and guanidine bases as initiators in the ring-opening polymerization of *N*-carboxyanhydrides.

The polymerizations of NCA of L-glutamic acid γ-benzyl ester (NCA_{Glu}) in DMF and DCM using DBU as initiator were very fast. Within 5 s, all monomer had been consumed, and the final molecular weight was obtained. Copolymers of NCA_{Cys} or NCA_{PhAla} and NCA_{Glu} were also prepared successfully using the same procedures, and the copolymers exhibited similar characteristic as the polypeptides of NCA_{Glu}. The fast polymerization rates that were obtained using DBU as initiator gave rise to highly robust polymerizations. The polymerizations could be performed without an inert atmosphere and without purification or drying of solvents. No extensive purification of the NCA monomers was needed and the polymerizations were not sensitive to a large amount of water present in the reaction mixture.

The *in situ* polymerization of N-carboxyanhydrides using DBU during spray-drying gave rise to spherical particles with sizes around ~1 μm. The particle size exhibited a dependence on the monomer feed concentration, where a lower concentration gave rise to smaller particles. The best results were obtained using $CHCl_3$ as solvent and a concentration of 2.5 mg/mL.

There are several advantages with *in situ* polymerization during spray-drying compared to performing the 2 steps separately. First, this pathway is "greener" as it combines 2 steps, reduces the amount of solvent needed and requires little purification. Second, most polypeptides have limited solubility in organic solvents, making it difficult to obtain spherical particles as indicated by the collapsed particles prepared by spray-drying of separately prepared PGlu. Third, particles of poly(S-benzyl-cysteine) could be prepared with this method in contrast to the 2 step preparation (polymerization followed by spray-drying) due to the poor solubility of poly(S-benzyl-cysteine).

We have here shown a straightforward method for obtaining polypeptide particles via an *in situ* polymerization during spray-drying. This furthers the use of spray-drying and enables the use of polypeptide particles for biomedical applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.6b00747.

^{1}H- and ^{13}C-NMR spectra, further characterization of the prepared polymers, SEM micrographs can be found in the Supporting Information (PDF)

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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