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Synthesis of Poly(Asparagine-*co*-Phenylalanine)
Copolymers, Analogy with Thermosensitive
Poly(Acrylamide-*co*-Styrene) Copolymers and
Formation of PEGylated Nanoparticles

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Abstract

We presented optimized synthetic pathways to high purity L-phenylalanine (Phe) and L-asparagine (Asn) derived *N*-carboxyanhydrides (NCA) in one-pot processes and in a highly reproducible manner. Ring-opening homopolymerizations of Asn-NCA and Phe-NCA were successfully achieved together with their copolymerization in an attempt to mimic upper critical solution temperature (UCST) vinylic poly(acrylamide-*co*-styrene) (P(Aam-*co*-St)) copolymers. Poly(asparagine-*co*-phenylalanine) (P(Asn-*co*-Phe)) copolypeptides of different Asn-to-Phe ratio were prepared and their thermoresponsive behavior was investigated in aqueous solution. Unfortunately, polymer aggregation imparted by strong hydrophobic interactions favored due to presence of β -sheet structures prevented achieving thermosensitive response in aqueous media in the 50-62°C range conversely to its vinylic counterpart. However, by taking advantage of those strong hydrophobic interactions, PEG-*b*-PAsn and PEG-*b*-PPhe diblock copolymers were prepared from amino-PEG initiators and PEG-*b*-PAsn was successfully formulated into PEGylated nanoparticles of ~270 nm.

Keywords

N-carboxyanhydride, Asparagine, Phenylalanine, Polypeptide, Nanoparticles, UCST

1. Introduction

Synthetic poly(amino acid)s, commonly known as polypeptides constitute a well-established class of biodegradable and biocompatible polymers covering a wide range of applications, from drug delivery, tissue engineering to biosensors.[1] Thanks to their unique amino-acid sequence and composition, these bio-inspired materials possess unique properties such as the possibility to self-assemble *via* non-covalent interactions or to endorse specific conformations such as α -helices or β -sheets.[2] For years, solid phase peptide synthesis (SPPS) has been the technique of choice to prepare synthetic polypeptides.[3] Although very attractive to obtain polypeptides with specific amino-acid sequences, this tedious method based on multiple coupling/deprotection steps usually prevents high molecular weight polypeptides to be obtained.[4] Ring-opening polymerization (ROP) of α -amino acid *N*-carboxyanhydrides (NCAs) initiated by nucleophiles is another widely known technique to synthesize polypeptides that, conversely to SPPS, enables achieving high molecular weights.[5,6] **Controlled polymerization processes of NCA monomers enable to access a wide variety of novel and innovative polypeptidic biomaterials with unique properties for targeted applications.**[7–9] As such, designing novel NCAs as building blocks to prepare polypeptide-based materials with new properties arouse considerable attention.

Polypeptides have indeed particularly gained an increase interest in the field of stimuli-responsive polymeric materials.[10–14] Such materials, that are able to change their physico-chemical properties upon external stimulus, such as pH, light, electric/magnetic fields, mechanical force, and more particularly temperature, have been thoroughly studied over the past years. In the field of thermoresponsive polymers, researchers have aimed at designing polypeptidic analogues of well-known non-degradable thermoresponsive vinylic polymers. For example, polypeptide obtained by ROP of **OEGylated L-glutamate** NCAs analogous of oligo(ethylene glycol) (OEG) methacrylate (OEGMA) have been developed as

alternative thermoresponsive materials with tunable lower critical solution temperature (LCST).[15,16] Inspired by poly(allylurea-*co*-allylamine) upper critical solution temperature (UCST) copolymers, Maruyama *et al.* prepared thermoresponsive poly(ornithine-*co*-citrulline) copolypeptides that mimic ureido groups of NCA derived from L-ornithine α -amino-acids.[17,18] More recently, Argawal *et al.* reported poly(acrylamide-*co*-styrene) (P(AAm-*co*-St)) copolymers obtained by reversible addition fragmentation chain transfer polymerization (RAFT) exhibiting sharp UCST behavior in water in the range of 50-62°C.[19] The synthesis of structurally analogous polypeptides with pendant amide and phenyl functionalities of P(AAm-*co*-St) has, however, never been attempted. In the broad family of amino acids, L-asparagine and L-phenylalanine possess amide and phenyl groups that structurally resemble acrylamide and styrene repeating units, respectively. Despite this evidence, literature on the copolymerization of L-asparagine and L-phenylalanine derived NCAs (Asn-NCA and Phe-NCA, respectively) to prepare poly(asparagine-*co*-phenylalanine) (P(Asn-*co*-Phe)) copolypeptides is lacking. While polypeptides composed of Phe-NCA have been extensively studied in the literature, successful preparation of poly(asparagine) only relies on the aminolysis of poly(succinimide).[20–29]

In the literature, two different carbonylation pathway prevailed for the preparation of NCA monomers.[30] The first one, called the Fuchs-Farthing method, involved the phosgenation **under heating conditions** of unprotected α -amino acids.[31,32] The second one, which derived from the Leuchs method, refers to the reaction of urethane-protected α -amino acids with **halogenating agents including** phosphorous halides, such as PBr₃ and PCl₅. [33–35] In both cases, the intended NCAs are obtained with the generation of HCl or phosphoric acid respectively, as side products. These acids not only lower the reaction kinetic but can also cause the undesired ring opening reaction of the NCAs to generate acyl chloride or acyl phosphate which consequently yields lower purity monomer.[36–40] Several approaches have

already been described in the literature to overcome these drawbacks, such as the use of an acid scavenger including tertiary amines or alkenes, vacuum or inert gas flow conditions.[39,41–47] The most popular route to date remains the use of an acid scavenger. Several methodologies have been presented for the synthesis of Phe-NCA.[21–28,39,48–51] Up to date, the reaction of L-phenylalanine with triphosgene in anhydrous THF under inert atmosphere at 50°C without any acid scavenger sometimes followed by tedious multiple recrystallization steps prevails.[21–26,48] As for synthetic Asn-NCA, **although commercially available**, only one example can be found in the literature. In this early attempt, benzyloxycarbonyl-L-asparagine was treated with PBr₃ in dioxane at room temperature to afford Asn-NCA in 35 % yield upon purification by column chromatography.[35] The authors pointed out, however, the poor yields obtained upon using this anhydride in peptide synthesis. **Therefore, there is a need for an optimized synthetic pathway for this monomer.**

Our motivation in this study was to: (i) develop reliable, sturdy and optimized synthetic pathways for the synthesis of both Phe-NCA and Asn-NCA; (ii) prepare for the first-time poly(asparagine-*co*-phenylalanine) (P(Asn-*co*-Phe)) copolypeptides; (iii) probe their potential thermoresponsivity in water in analogy with poly(acrylamide-*co*-styrene) copolymers and (iv) prepare amphiphilic PEG-*b*-PAsn and PEG-*b*-PPhe copolymer nanoparticles (**Fig. 1**).

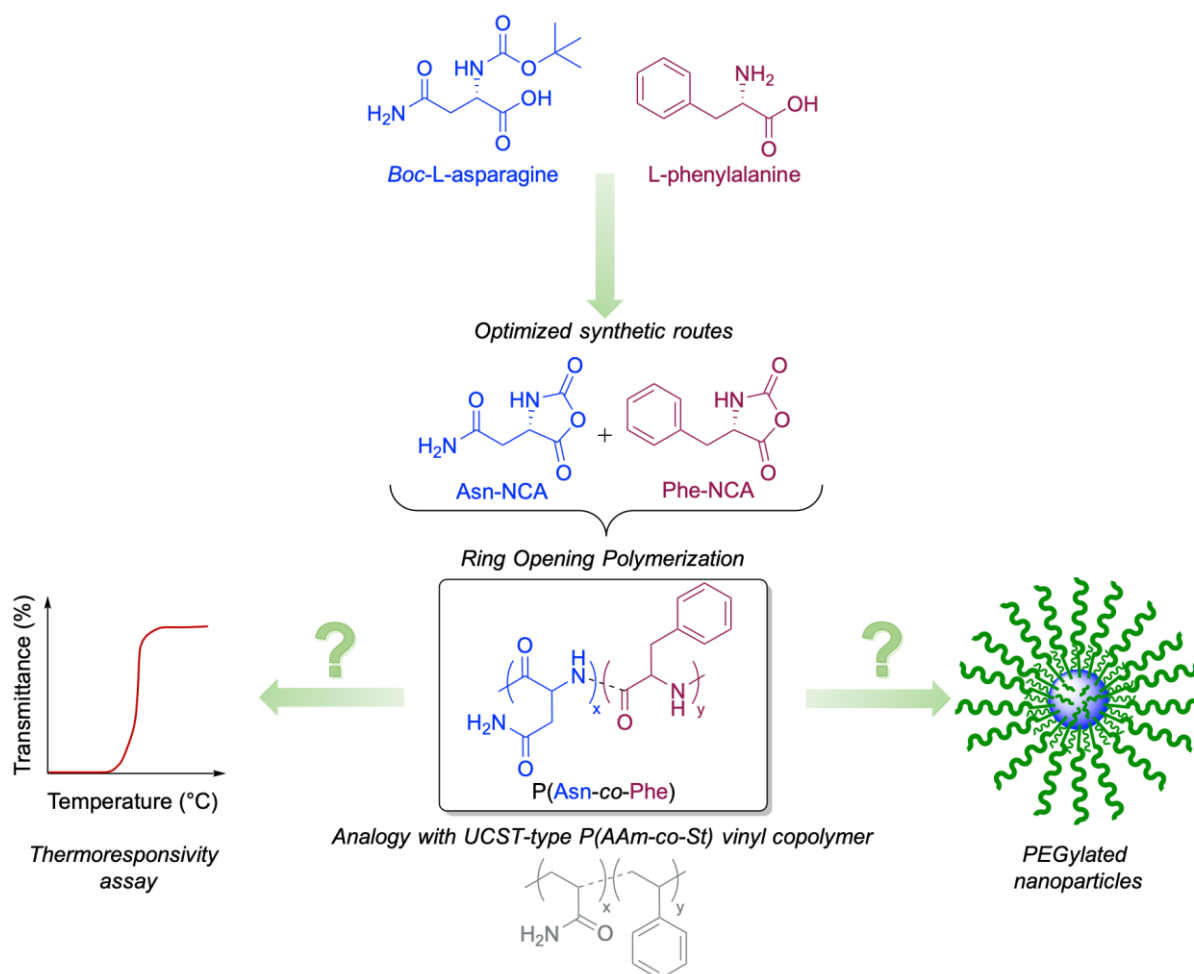


Fig 1. Preparation of poly(asparagine-*co*-phenylalanine) (P(Asn-*co*-Phe)) copolypeptides by ROP of L-asparagine and L-phenylalanine-derived *N*-carboxyanhydrides obtained from optimized synthetic pathways and potential applications envisioned.

2. Experimental part

2.1. Materials

L-phenylalanine (>98.0 %), neopentylamine (>98.0 %), *N*- α -(*tert*-Butoxycarbonyl)-*N*- γ -trityl-L-asparagine (Boc-L-Asn(Trt)-OH, >98.0 %), *N*- α -(*tert*-Butoxycarbonyl)-L-asparagine (Boc-L-Asn-OH, >98.0 %), *N*-methylmorpholine (NMM, >99.0 %), (1*R*)-(+)- α -pinene (>97.0 %), triisopropylsilane (>98.0 %) and triphosgene (>98.0 %) were purchased from TCI chemicals. Triethylamine (TEA, 99 %), trifluoroacetic acid (TFA, 99 %), phosphorous tribromide (PBr₃) solution (1 M in DCM), anhydrous solvents THF (\geq 99.9%, inhibitor-free), DMF (99.8%),

DCM ($\geq 99.8\%$, contains 40-150 ppm amylene as stabilizer), EtOAc (99.8 %) were purchased from Sigma Aldrich. Methoxypolyethylene glycol amine (PEG_{5k}-NH₂, $M_n = 5,516$ Da) was purchased from Iris Biotech. *n*-Hexane (technical grade) was purchased from VWR. Diethyl ether (99.8 %) was purchased from Carlo Erba. Deuterated solvents such as CDCl₃, DMSO-d₆ was purchased from Eurisotop. All materials were used without further purification. All manipulations were performed under moisture and oxygen-free conditions using conventional Schlenk techniques.

2.2. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy was performed in 5 mm diameter tubes in DMSO-d₆ at 25 °C. ¹H and ¹³C NMR spectroscopy was performed on a Bruker Avance 300 spectrometer at 300 MHz (¹H) and 75 MHz (¹³C), respectively. The chemical shift scale was calibrated based on the internal solvent signals ($\delta = 2.50$ ppm for DMSO-d₆ and $\delta = 7.26$ ppm for CDCl₃). Data were reported as: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad), coupling constants (J) given in Hertz (Hz), and integration.

2.3. Fourier transform infrared (FTIR) spectroscopy

FT-IR spectra were obtained by FT-IR spectrophotometer (Spectrum One, PerkinElmer, USA) using attenuated total reflectance (ATR) technique. Spectra were recorded between 4000-525 cm⁻¹ with a spectrum resolution of 4 cm⁻¹. All spectra were averaged over 10 scans.

2.4. Elemental analysis

The elemental analysis for carbon, hydrogen and nitrogen content determination was performed using a Perkin Elmer 2400 series elemental analyzer (PerkinElmer, USA).

2.5. Dynamic Light Scattering (DLS) and zeta potential

Nanoparticle diameters (Dz) and particle size distribution (PSD) were measured by dynamic light scattering (DLS) with a Nano ZS from Malvern equipped with a 4 mW He–Ne laser (633 nm wavelength) at a fixed scattering angle of 173° and temperature of 25°C. The surface charge of the NPs was investigated by ζ -potential (mV) measurement at 25 °C after dilution with 1 mM NaCl, using the Smoluchowski equation.

2.6. Synthesis of L-phenylalanine N-carboxyanhydride (Phe-NCA)

Synthesis of Phe-NCA was carried out in an adapted version of Daly *et al.*[37] In a 250 mL round bottom flask equipped with a magnetic stirrer, L-phenylalanine (1 eq., 0.061 mol, 10 g) and α -pinene (2.1 eq., 0.12 mol, 19,2 mL) were added into 100 mL of anhydrous THF (0.6 M total). An additional funnel was affixed to the round bottom flask and charged with 6.65 g of triphosgene (0.37 eq., 0.02 mol, 6.65 g) and 30 mL of anhydrous THF. The setup was heated up to 50°C for 10 min. The triphosgene–THF mixture was dripped into the round bottom flask over a period of 15 min. After the addition of triphosgene, the reaction was stirred for an additional 2 h at 50°C at which time most of the L-phenylalanine had disappeared. The reaction mixture was filtered to remove the undissolved L-phenylalanine. The filtrate was then concentrated to 1/3 of its initial volume under vacuum and then added dropwise into excess cold *n*-hexane. The flask was placed at -20°C for 24 h to allow complete precipitation of the product. The white powder was then dried under vacuum at room temperature overnight (7.74 g, 67 % yield). ¹H NMR (300 MHz, CDCl₃): 7.38-7.29 (m, 3H); 7.20-7.17 (m, 2H); 6.14 (bs, 1H); 4.55-4.51 (dd, *J* = 8.7 Hz, 4.1 Hz, 1H); 3.25-3.31 (dd, *J* = 14.1 Hz, 3.9 Hz, 1H); 3.04-2.96 (dd, *J* = 14 Hz, 8.7 Hz, 1H). ¹³C NMR (75 MHz, CDCl₃): 169, 152.3, 134, 129.4, 129.3, 128, 59, 37.8. IR (ATR, cm⁻¹): 3258, 1850, 1754, 1492, 1453, 1364, 1329, 1276,

937. Anal. Calcd for C₁₀H₉NO₃: C, 62.82; H, 4.74; N, 7.33; O, 25.11. Found: C, 61.92; H, 4.72; N, 7.19. Characterization data are consistent with previous reports.[25,39,47,49–51]

2.7. Synthesis of *N*- γ -trityl-*L*-asparagine *N*-carboxyanhydride (*Asn*(*Trt*)-*NCA*)

Synthesis of *Asn*(*Trt*)-*NCA* was carried out in an adapted version of Rao *et al.* To a suspension of Boc-*L*-*Asn*(*Trt*)-OH (1 eq., 5 mmol, 2.4 g) in dichloromethane (50 mL, 0.1 M total) under N₂ was added PBr₃ (0.6 eq., 3 mmol, 3 mL) dropwise. α -pinene (3.5 eq., 18 mmol, 2.78 mL) was immediately added dropwise. The suspension turned to pale yellow and about 10 min later all the starting material had dissolved. The mixture was stirred at ambient temperature for 24 h. The solid was filtered, rinsed with dry DCM and dried under vacuum to give *Asn*(*Trt*)-*NCA* as a white powder (1.59 g, 80 % yield). ¹H NMR (300 MHz, DMSO-d₆): 8.97 (s, 1H); 8.86 (s, 1H); 7.14-7.30 (m, 15H); 4.53-4.56 (t, *J* = 3.7 Hz, 1H); 3.03-3.10 (dd, *J* = 16.4 Hz, 4.4 Hz, 1H); 2.72-2.79 (dd, *J* = 14.4 Hz, 3.5 Hz, 1H). ¹³C NMR (75 MHz, DMSO-d₆): 171.4, 167.7, 152.2, 144.4, 128.5, 127.5, 126.4, 69.6, 53.9, 36.7. IR (ATR, cm⁻¹): 3414, 3357, 1858, 1787, 1665, 1508, 1495, 1447, 1401, 1366, 1332, 1284, 1267, 908, 901. Characterization data are consistent with previous report.[52]

2.8. Deprotection of *N*- γ -trityl-*L*-asparagine *N*-carboxyanhydride (*Asn*-*NCA*, route A)

In a 25 mL round bottom flask equipped with a magnetic stirrer, *Asn*(*Trt*)-*NCA* (1 eq., 0.3 g, 0.75 mmol) was dissolved in neat anhydrous TFA (7.6 mL) under stirring. Triisopropylsilane (10 eq., 7.5 mmol, 1.5 mL) was then added and a white solid appeared (trityl). The whole mixture was allowed to stir at room temperature under N₂ atmosphere for 10 min. Then, the reaction mixture was filtered, concentrated under vacuum and then added dropwise to 10-fold cold THF (-20°C) where a white precipitate appeared. The whole was centrifuged at 10,000 rpm at 10 °C for 10 min. After discarding the liquid fraction, new THF was added, and the

product was suspended in a sonic bath. The suspension was centrifuged again, and the procedure was repeated two times to give Asn-NCA as a white powder (0.39 g, 33 % yield). ^1H NMR (DMSO- d_6): 8.78 (s, 1H); 7.50 (s, 1H); 7.05 (s, 1H); 4.50-4.53 (t, $J = 4.1$ Hz, 1H); 2.59-2.73 (ddd, $J = 21.5$ Hz, 16.9 Hz, 4.5 Hz, 2H). ^{13}C NMR (75 MHz, DMSO- d_6): 171.7, 169.8, 152.5, 53.7, 35.5. IR (ATR, cm^{-1}): 3417, 3187, 1835, 1791, 1778, 1674, 1659, 1435, 1425, 1389, 1366, 1297, 1270, 940, 912.

2.9. Synthesis of *L*-asparagine *N*-carboxyanhydride (Asn-NCA, route B)

To a suspension of Boc-*L*-Asn-OH (1 eq., 10 mmol, 2.32 g) in THF (150 mL, 0.06 M total) under Ar was added PBr_3 (0.6 eq., 6 mmol, 6 mL) dropwise. α -Pinene (4 eq., 40 mmol, 6.35 mL) was immediately added dropwise. The suspension turned to pale white and about 10 min later all the starting material had dissolved. The mixture was stirred at ambient temperature for 24 h. The reaction mixture was then filtered, and the filtrate concentrated under vacuum. THF was then added and a white precipitate appeared. The solid obtained was filtered, washed with THF and dried under vacuum to give Asn-NCA as a white powder (0.9 g, 57 % yield). ^1H NMR (300 MHz, DMSO- d_6): 8.79 (s, 1H); 7.51 (s, 1H); 7.05 (s, 1H); 4.50-4.53 (t, $J = 4.1$ Hz, 1H); 2.59-2.74 (ddd, $J = 21.9$ Hz, 16.9 Hz, 4.9 Hz, 2H). ^{13}C NMR (75 MHz, DMSO- d_6): 171.7, 169.9, 152.5, 53.7, 35.5. IR (ATR, cm^{-1}): 3417, 3186, 1843, 1780, 1766, 1683, 1670, 1434, 1401, 1353, 1295, 1270, 941, 908. Anal. Calcd for $\text{C}_5\text{H}_6\text{N}_2\text{O}_4$: C, 37.98; H, 3.82; N, 17.72; O, 40.48. Found: C, 38.2; H, 3.93; N, 16.75.

2.10. General procedure for the ROP of Asn(*Trt*)-NCA and Phe-NCA ($[M]/[I] = 25$)

The P(Asn(*Trt*)_{22.5}-*co*-Phe_{2.5}) polypeptide was prepared following conventional ROP procedure. Asn-*Trt*-NCA (90 mol.%, 22.5 eq., 0.63 mmol, 0.25 g) and Phe-NCA (2.5 eq., 0.07 mmol, 13.4 mg) (total mole = 0.7 mmol) were loaded into a flame-dry Schlenk tube

equipped with a magnetic stirrer. The Schlenk tube was purged three times with argon/vacuum cycles. Then, 1.16 mL of dry DMF was added (0.6 M). For initiation, a total of 3.3 μ L of neopentylamine (1 eq., 0.03 mmol) was directly added to the Schlenk tube via syringe. The reaction was let under stirring at 30°C for 4 days. Directly after completion of the reaction, the polymer was precipitated in cold diethyl ether and centrifuged at 10,000 rpm at 10 °C for 10 min. After discarding the liquid fraction, new ether was added, and the polymer was suspended in a sonic bath. The suspension was centrifuged again, and the procedure was repeated two times. The obtained protected copolymer was then dried under vacuum overnight.

2.11. Deprotection of P(Asn(Trt-co-Phe)

Deprotection of the protected copolymer was carried out similarly to the deprotection of Asn(Trt)-NCA (Asn-NCA, route A). Briefly, the copolymer was dissolved in neat TFA under stirring. Excess triisopropylsilane was then added and the mixture was allowed to stir for 10 min. The reaction mixture was then filtered, concentrated under vacuum and then added dropwise to 10-fold cold THF (-20°C) where a white precipitate appeared. After discarding the liquid fraction, new THF was added, and the product was suspended in a sonic bath. The suspension was centrifuged again, and the procedure was repeated two times.

2.12. General procedure for the ROP of Asn-NCA and Phe-NCA ([M]/[I] = 50)

The P(Asn₄₅-co-Phe₅) polypeptides was prepared as follow. Asn-NCA (45 eq., 1.35 mmol, 0.21 g) and Phe-NCA (5 eq., 0.15 mmol, 0.03 g) (total mole = 1.5 mmol) were loaded into a flame-dry Schlenk tube equipped with a magnetic stirrer. The Schlenk tube was purged three times with argon/vacuum cycles. Then, 25 mL of dry DMSO (0.06 M) was added. For initiation, a total of 3.5 μ L of neopentylamine was directly added to the Schlenk tube via

syringe. The reaction was let under stirring at 30°C for 4 days. Directly after completion of the reaction, the polymer was precipitated to cold THF and centrifuged (10,000 rpm at 10 °C for 10 min). After discarding the liquid fraction, new THF was added, and the polymer was suspended in a sonic bath. The suspension was centrifuged again, and the procedure was repeated two times. The obtained copolymer was then dried at room temperature under vacuum overnight. Similar polymerization conditions were applied for the P(Asn₄₀-co-Phe₁₀) and P(Asn₄₉-co-Phe₁) polypeptides.

*2.13. General procedure for the synthesis of PEG-*b*-PNCA diblock copolymer ([M]/[I] = 60)*

Asn-NCA (60 eq., 2 mmol) was loaded into a flame-dry Schlenk tube equipped with a magnetic stirrer. The Schlenk tube was purged three times with argon/vacuum cycles. Then, 3 mL of anhydrous DMSO was added. For initiation, a total of 165 mg of PEG_{5k}-NH₂ (0.03 mmol) in 2 mL DMSO was directly added to the Schlenk tube via syringe (0.4 M total). The reaction was let under stirring at 30°C for 4 days. Directly after completion, the polymer was precipitated in cold THF and centrifugated at 10,000 rpm et 5°C for 10 min. After discarding the liquid fraction, new THF was added, and the polymer was suspended in a sonic bath. The suspension was centrifuged again, and the procedure was repeated two times. The obtained diblock polymer was then dried at room temperature under vacuum overnight. Similar polymerization conditions were applied for the PEG-*b*-PPhe polypeptide.

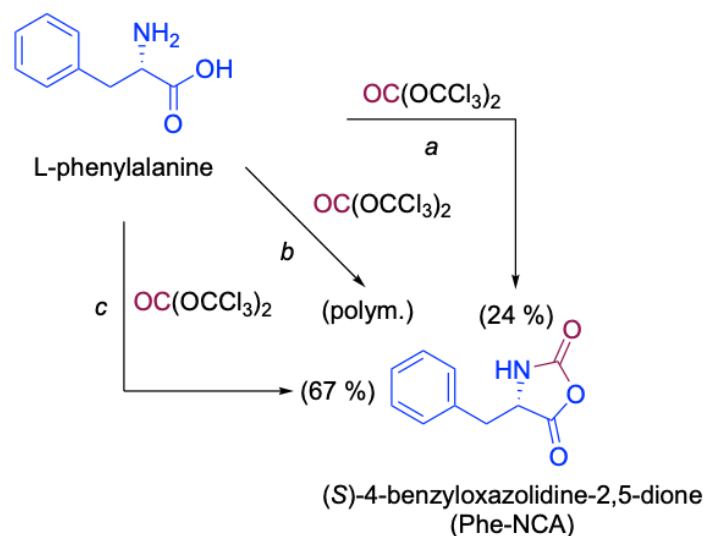
2.14. Nanoparticles preparation

Nanoparticles were prepared from the PEG-*b*-PASn and PEG-*b*-PPhe diblock copolymers. Nanoprecipitation was performed by the dropwise addition (1 mL.min⁻¹) of the polymer solution (10 mg.mL⁻¹ in TFA, 2 mL) into vigorously stirred (700 rpm) deionized water (20 mL). TFA was then gently removed under vacuum before DLS analysis.

3. Results and Discussion

3.1. Synthesis and characterization of NCA monomers

Research for optimal acid scavenger which can be used in both carbonylation pathways describe in the introduction that allows easy recovery of high purity NCA with good yields and in a highly reproducible manner was initially carried out using L-phenylalanine as the model α -amino-acid (**Scheme 1**). Reaction of L-phenylalanine with triphosgene in anhydrous THF under inert atmosphere at 50°C was carried out using three different scavengers of acid byproducts that have proven effective for NCA synthesis in peculiar conditions: (i) the use of Ar flow in NaOH solution; (ii) the use of *N*-methylmorpholine as a weak base and (iii) the use of α -pinene.[38,39,41,46,49–51]



Scheme 1. Synthetic pathways to (*S*)-4-benzyloxazolidine-2,5-dione (Phe-NCA). Reagents: (a) Ar flow in NaOH solution (10 mM); (b) *N*-methylmorpholine (4.5 equiv.); (c) α -pinene (2.1 equiv.).

Using argon flow to trap HCl in a 0.1 M sodium hydroxide solution product lead to poor monomer recovery (24 % yield) whether the reaction was performed under concentrated (0.6

M) or diluted (0.06 M) conditions (**Scheme 1.a**). Similar behavior was obtained without any acid scavenger. In a recent study, Fuse and coworkers who prepared a library of NCAs in a micro flow reactor, using basic-to-acidic flash switching, have shown that *N*-methyl morpholine (NMM) was a suitable base to trap HCl without deprotonating NCA.[39] When applied to our system, however, the use of NMM prevented to achieve controlled and efficient synthesis of Phe-NCA as polymerization occurred (**Scheme 1.b**). Consequently, we broadened our investigations with the use of terpenes as efficient scavengers of acid byproducts. In a patent from 2002, the use of alkenes for the chemical removal of HCl was reported.[41] A few years later, Hulshof *et al.* demonstrated a similar application of alkenes for the synthesis of L-leucine NCA, where, two different terpenes were selected, *i.e.* *S*-(-) α -pinene and *R*-(+)-limonene.[38] α -pinene has already been successfully employed as acid scavenger for the synthesis of Phe-NCA, although the authors performed the reaction in harsh conditions (reflux) and multiple recrystallization steps were applied to achieve pure monomer.[49–51] When implemented to our synthetic procedure, pure Phe-NCA monomer was similarly obtained in a good yield of 67 % but surprisingly without requiring multiple recrystallization procedures (**Fig 2.a** and **Fig. S1-S2** in ESI). It is worth pointing out that the introduction of an alkene as HCl scavenger into the reaction mixture resulted in the formation of pinene hydrochloride byproducts, also named bornyl chloride. Nevertheless, this component was easily washed away during precipitation in *n*-hexane. While the yield obtained was in the same range as those described in the literature, this procedure – which does not require purification steps – offered better reliability as yield achieved without acid scavengers often depends on the multiple recrystallization efficiency.[21,37]

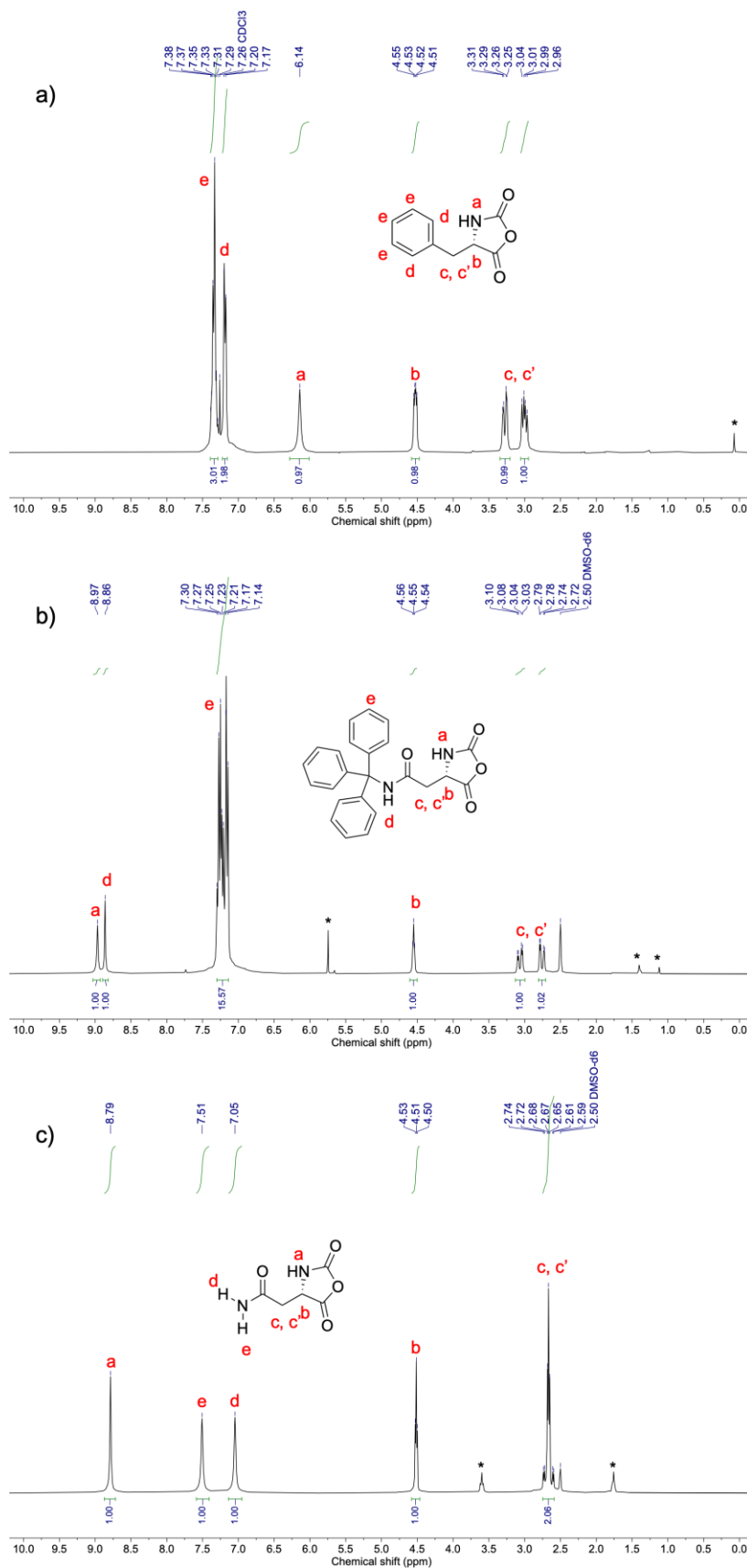
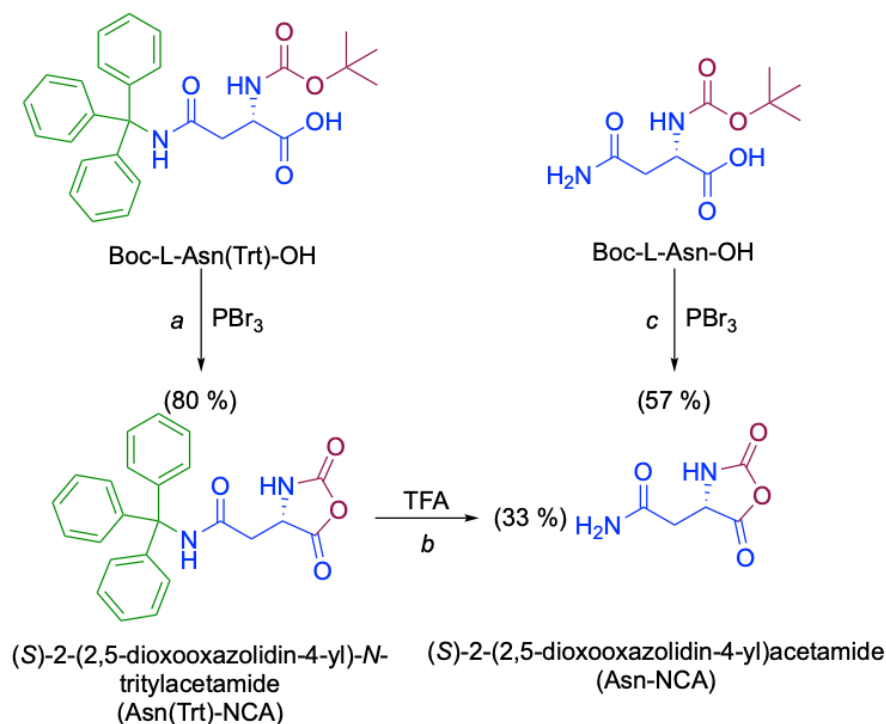


Fig 2. ¹H NMR spectra of (a) Phe-NCA (CDCl₃), (b) Asn(Trt)-NCA (DMSO-d₆) and (c) Asn-NCA (DMSO-d₆). Residual solvent traces have been noted by *.

Because phosgene reacts with primary amide to yield nitrile, Asn-NCA cannot be prepared by the direct phosgenation of the corresponding L-asparagine amino acid.[53,54] Moreover, asparagine-containing peptides suffer from low solubility in organic solvent as well as aggregation phenomenon due to numerous hydrogen bonds. Aiming to improve monomer synthesis as well as peptides formation, several substituents and notably trityl (Trt) group have been used as protecting carboxamide groups.[55] We envisioned two synthetic pathways to access Asn-NCA (**Scheme 2**). The first one involved the preparation of Asn(Trt)-NCA modelled on literature procedure (*i.e.* reaction of Boc-L-Asn(Trt)-OH with PBr₃ in DCM in the presence of triethylamine) followed by its deprotection under acidic condition (**Scheme 2.a** and **b**).[52] Despite the reaction being described as quantitative, this synthetic route employing triethylamine as acid scavenger could not result in the isolation of pure monomer, as triethylamine acid salt precipitated out together with the product. In light of the encouraging results obtained with α -pinene as acid scavenger for the preparation of Phe-NCA, Boc-L-Asn(Trt)-OH was reacted with PBr₃ in the presence of 2.1 equiv. of the terpene. The purity of the monomer obtained was, however, altered by the formation of undesired byproduct as observed by NMR spectroscopy with signals at $\delta = 7.78$ and 5.66 ppm. Employing 3.5 equiv. of α -pinene resulted in increased monomer purity up to 97% while maintaining the yield to about 80 % (**Fig 2.b** and **Fig. S3-S4** in ESI). Overall, the synthesis of Asn(Trt)-NCA provides an improvement on the previously reported synthetic pathway, as the NCA could be recovered in high yield and high purity without traces of triethylammonium salt.



Scheme 2. Synthetic pathways to (*S*)-2-(2,5-dioxooxazolidin-4-yl)acetamide (Asn-NCA). Reagents/Methods: (a) α -pinene (3.5 equiv.); (b) triisopropylsilane (10 equiv.); (c) α -pinene (4 equiv.).

The Asn(Trt)-NCA was then deprotected in neat anhydrous TFA at room temperature under argon (**Scheme 2.b**).^[56] Performing the acidolysis of Asn(Trt)-NCA in the absence of carbocation scavenger led to no evidence of successful removal of trityl protecting group. Anisole or organosilicon compounds, such as, triethylsilane are well known carbocation scavengers for the selective removal of protecting groups by acidolysis, such as Boc and related *tert*-butyl groups.^[57–59] While using a mixture of TFA/anisole (96/4 % v/v), only partial deprotection of the trityl group was observed, whilst employing 10 equiv. of triisopropylsilane led to quantitative yield. In addition, the work-up procedure was very straightforward as the protonated trityl group precipitated out in TFA, which after filtration allowed an easy recovery of Asn-NCA upon precipitation in diethyl ether (33 % yield). Clean and complete removal of the trityl protecting group was deduced from the disappearance of all the aromatic signals from the ¹H NMR at $\delta = 7.14\text{--}7.30$ ppm. The appearance of two new

signals associated to the deprotected amide was observed at $\delta = 7.50$ and 7.05 ppm respectively (**Fig. S5-S8** in ESI). Further confirmation was obtained from the change in solubility of the resulting monomer from Asn(Trt)-NCA, soluble in THF, to Asn-NCA, insoluble in THF.

The second synthetic pathway envisioned to achieve Asn-NCA was an improved methodology based on Denkewalter and coworkers' procedure, which notably increased the overall yield without requiring column chromatography purification (**Scheme 2.c**).^[35] Applying our optimized Asn(Trt)-NCA synthetic procedure starting from *N*- α -Boc-L asparagine failed due to insolubility of both *N*- α -Boc-L asparagine and its corresponding NCA in DCM. As a consequence, the resulting Asn-NCA was formed in low yield, with approximately 30 % of unreacted *N*- α -Boc-L asparagine and a significant amount of hydrolysis product that could clearly be observed by ¹H NMR (**Fig. S9** in ESI). Changing the solvation conditions by employing THF instead of DCM proved effective as reaction reached completion but again suffered from partial hydrolysis as traces of the analogous α -amino acid could be seen on ¹H NMR. Addition of a larger excess of α -pinene (4 equiv.) has proven to be beneficial as it prevented hydrolysis, but the reaction yield remained low (22 %) and thus, did not provide an improvement on the previously reported synthetic pathway. It has already been shown that introduction of alkenes into the reaction mixture lowers the overall polarity of the medium and consequently the reaction rate yielding to poor monomer recovery. Thus, in the pursuit of our effort to optimize our synthetic route, we then investigated the effect of concentration by performing the reaction under dilute conditions (0.06 M). This eventually provided considerable improvement, as crystalline Asn-NCA could be obtained in 57 % yield after precipitation in THF, which is almost three times the one obtained in concentrated medium (**Fig 2.c** and **Fig. S10-S11** in ESI). Overall, this optimized synthetic pathway gives several key improvements over the other reported methods: (i) the use of low-cost and potent

acid scavenger; (ii) work-up procedure have been greatly reduced as no recrystallization or column chromatography are needed and (iii) Asn-NCA is obtained in high yield.

After developing enhanced synthetic procedures based on the utilization of α -pinene as effective acid scavenger to access acrylamide-like Asn-NCA and styrene-like Phe-NCA, assay to copolymerize these monomers to form potentially thermosensitive copolypeptides was then performed for the first time.

3.2. Ring-opening polymerization studies

As shown by Agarwal *et al.*, vinylic copolymers obtained by copolymerization between St and AAm possessed a rather narrow composition window, ranging from 14 to 16 mol.% of styrene, in which they exhibit a UCST.[19] Inspired by these results, we initially decided to introduce in the feed 90 mol. % of Asn(Trt)-NCA and 10 mol. % of Phe-NCA to yield P(Asn(Trt)-*co*-Phe) (**Table 1, entry 1**). However, attempts to prepare P(Asn(Trt)-*co*-Phe) at 30°C in anhydrous DMF using neopentylamine as initiator ($[M]/[I] = 25$) were partially successful. The copolymerization gave rather oligopeptides, most likely due to restricted chain extension during polymerization caused by: (i) the high steric hindrance induced by the bulky trityl protective group and (ii) the occurrence of β -sheet secondary structures imparted by numerous intermolecular hydrogen bond interactions (see section 3.3 and Fig. 4 for details).[60] As a result, the deprotected copolymer obtained after acidolysis in TFA, was of lower molecular weight than expected as characterized by ^1H NMR spectroscopy ($M_n = 2 \text{ kg.mol}^{-1}$, $DP_n = 10$) (**Fig. S12-S13** in ESI).

Table 1. Experimental conditions and macromolecular characteristic of the copolymers prepared in this study.

Entry	Monomer (90 mol. %)	Comonomer (10 mol. %)	Solvent	[M] ₀ (M)	Temperature (°C)	Reaction time (h)	DP _{n,th}	DP _{n,NMR} ^a	M _{n,NMR} ^a (kg.mol ⁻¹)
1	Asn(Trt)- NCA	Phe-NCA	DMF	0.6	30	96	25	10	2
2	Asn-NCA	Phe-NCA	DMF	0.6	30	96	25	21	2.9
3	Asn-NCA	Phe-NCA	DMF	0.6	60	96	25	17	2
4	Asn-NCA	Phe-NCA	DMSO	0.6	30	96	25	17	2
5	Asn-NCA	Phe-NCA	DMSO	0.06	30	96	25	25	3

^a. Determined by ¹H NMR.

As such, our efforts then concentrated on performing the copolymerization between Phe-NCA and Asn-NCA instead of Asn(Trt)-NCA to directly yield P(Asn-co-Phe), as the less hindered non-protected monomer was postulated to lead to a more favorable polymerization. Adequate amide group protection is usually a requirement to prevent side reactions during peptide coupling synthesis from α -amino-acids such L-asparagine and L-glutamine.[55,61,62] Peptides obtained from these monomers often suffer from low solubility and aggregation caused by the numerous hydrogen bonds. However, by setting up proper polymerization conditions, we were able to minimize these inconveniences. We performed the ROP of Asn-NCA and Phe-NCA under identical experimental conditions as for entry 1 (**Table 1, entry 2**). As the neopentylamine initiator was added in one shot in the medium ([M]/[I] = 25), the reaction became translucent and finally turned a milky-white color within minutes. After 1 h, the reaction turned completely opaque. After precipitation of the polymer in cold THF, formation of the polypeptide was first assessed by ¹H NMR. It displayed the shifts of characteristic NCAs methylene protons (-CH₂-) adjacent to the α -methylene proton (-CH-)

resonances at $\delta = 2.59\text{-}2.74$ ppm and $\delta = 2.96\text{-}3.31$ ppm downfield towards new broader signal at $\delta = 3.37$ ppm. Meanwhile, the signals centered at $\delta = 4.51$ and 4.53 ppm (-CH- in monomers) disappeared as the reaction progressed, and a new broader triplet centered at $\delta = 4.49$ ppm (-CH- in polymer) appeared (**Fig. S14** in ESI). Neopentylamine end group analysis revealed that the targeted DP_n was nearly achieved (≈ 21). As a result of early precipitation during polymerization, the reactive polymer chain end was indeed not any longer accessible for further propagation, resulting in a slightly lower molecular weight polypeptide. Further characterization by FTIR-ATR provided additional evidence of the formation of polypeptide by the disappearance of the monomers C=O stretching vibration bands at 1850 and 1843 cm^{-1} and the appearance of a polypeptide C=O stretching band (amide I) at 1654 cm^{-1} (**Fig. S15** in ESI). Unfortunately, because the polymer obtained was insoluble in common SEC solvents such as DMF, THF, DMSO and CHCl_3 , it was impossible to measure neither number-average molecular weight ($M_{n,\text{SEC}}$) nor dispersity D , **that could be broad**. Different solutions have been studied to weaken hydrogen bonds and favor solubilization, such as, the use of heat or the addition of concentrated salts such as NaCl and KCl.[63,64] To avoid precipitation and achieve higher conversions, we investigated the effect of the polymerization temperature. Raising the temperature to 60°C still resulted in early precipitation and a polymer exhibiting a chain length of ≈ 17 was obtained (**Table 1, Entry 3** and **Fig. S16** in ESI). Recent studies have already reported that an increase in temperature generally inhibited polymerization due to the formation of *N*-formyl group at the polymer chain end resulting from the reaction between the propagating species and DMF.[27,28] Thus, we maintained the polymerization temperature to 30°C and switched solvent to DMSO, which is more polar than DMF. We believed that polymer-solvent interactions would be stronger than polymer-polymer intermolecular interactions, resulting in better solvation conditions while avoiding potential termination reactions. Again, we noted that $DP_{n,\text{NMR}}$ remained largely invariant of the

conditions (≈ 17) (**Table 1, Entry 4** and **Fig. S17** in ESI). However, the polymer behavior in solution drastically changed as no aggregation was observed during the first 72 h. Small aggregates could however be observed only at the end of polymerization. Despite being at the cost of reduced polymerization rate due to a decrease concentration of the propagating chains, the copolymerization between Asn-NCA and Phe-NCA initiated by neopentylamine was investigated by reducing the concentration by a factor of 10 (0.06 M) (**Table 1, Entry 5**). In doing so, not only we were able to fully suppress aggregation phenomenon but we also attained the targeted DP_n ($M_n = 3 \text{ kg}\cdot\text{mol}^{-1}$, $DP_n = 25$), with 90.2 % and 9.2 % of Asn and Phe units, respectively, as determined by ^1H NMR (**Fig. S18** in ESI).

3.3. Thermosensitivity assays

After optimizing the polymerization conditions, we tested potential UCST behavior of P(Asn-co-Phe). We synthesized three different copolymers using a range of Asn-NCA-to-Phe-NCA initial molar ratios (98:2; 90:10 and 80:20) initiated with neopentylamine at a monomer-to-initiator ratio of 50 ($[\text{M}]/[\text{I}] = 50$) (**Table 2**). End group analysis by ^1H NMR revealed polymer $DP_{n,s} \approx 38$ (98:2); 42 (90:10) and 45 (80:20) and molar masses M_n ranging from 4.2 to 5.4 $\text{kg}\cdot\text{mol}^{-1}$, based on the integration of the *tert*-butyl neopentylamine resonances at $\delta = 0.81$ ppm against those of the methylene -CH- protons from the main polymer chain at $\delta = 4.49$ ppm (**Fig. 3**). For %Asn and %Phe determination, we resulted again in the signals of the amide NH resonances at $\delta = 7.31$ -7.53 ppm and the signal of the Phe phenyl ring at $\delta = 7.14$ -7.31 ppm. The data obtained were in good correlation with the theoretical values for Asn-to-Phe ratios of 95:5 (98:2 in the feed), 90.5:9.5 (90:10 in the feed) and 85:15 (80:20 in the feed). Yet the technique was not completely accurate as signals from Asn amide NH and Phe phenyl ring tends to slightly overlap.

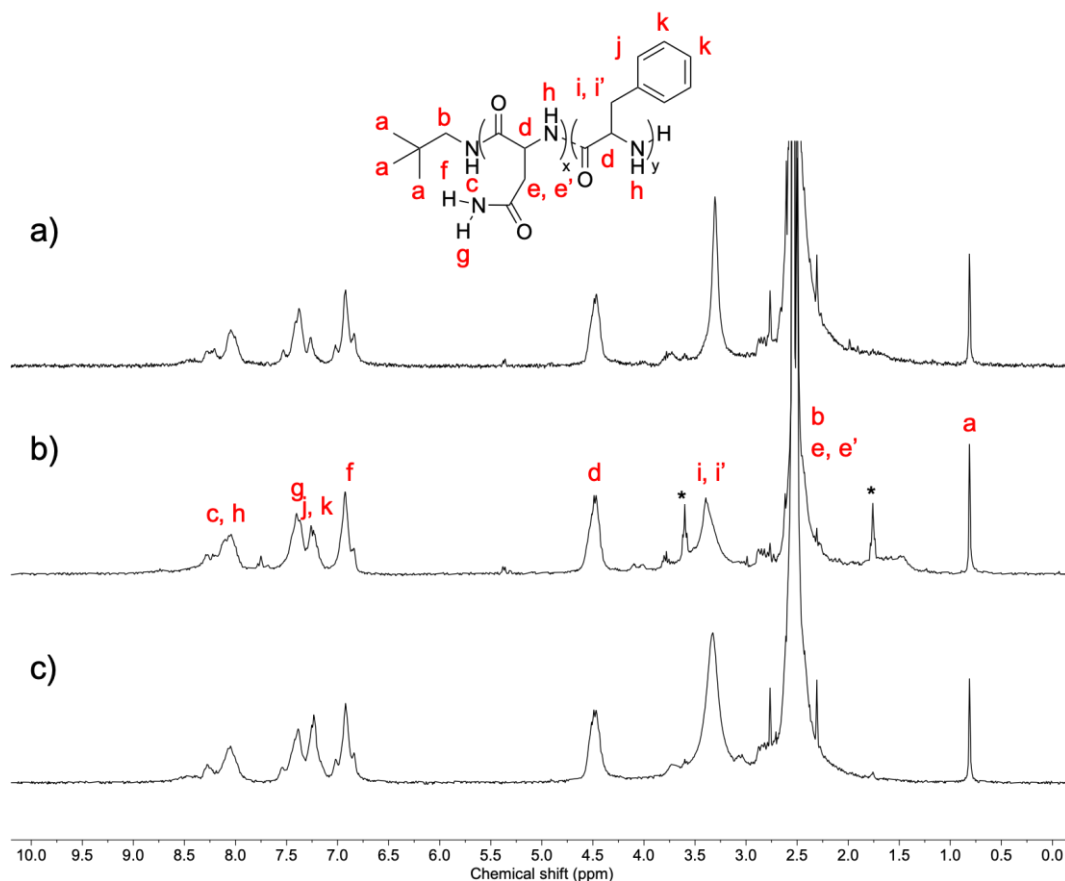


Fig 3. ^1H NMR in DMSO-d_6 of the $\text{P}(\text{Asn-co-Phe})$ copolymers with Asn-to-Phe molar ratio of: (a) 98:2; (b) 90:10 and (c) 80:20. Residual solvent traces have been noted by *.

Table 2. Macromolecular characteristics of $\text{P}(\text{Asn-co-Phe})$ copolymers prepared by ROP of Asn-NCA and Phe-NCA.

Entry	Feed (mol. %)		Polymer (%) ^a		Reaction time (h)	$DP_{n,\text{th}}$	$DP_{n,\text{NMR}}$ ^a	$M_{n,\text{NMR}}$ ^a (kg.mol ⁻¹)
	Asn-NCA	Phe-NCA	Asn	Phe				
1	98	2	95	5	96	50	38	4.2
2	90	10	90.5	9.5	96	50	42	5.2
3	80	20	85	15	96	50	45	5.4

^a. Determined by ^1H NMR.

The solubility in water of the copolymers obtained (1 wt.%) was then tested at different temperatures. As can be seen on the pictures (**Fig. S19** in ESI), none of the copolymers yielded a thermosensitive response in aqueous media (neither in deionized water nor PBS solution), independently of their composition or the temperature. Polymer suspensions obtained in both deionized water and PBS solution tended to precipitate after a few hours. In fact, the three copolymers were insoluble in most organic solvents, such as THF, DMSO, DMSO with 10 mM LiCl, DMAc, DMF, CHCl₃ and DCM. Only an aqueous solution of 75 % v/v TFA in water was able to solubilize the copolypeptides. The tendency of the copolymers to phase out in solution was probably due to the occurrence of strong β -sheet structures enhanced by the numerous intermolecular hydrogen bond interactions between amide NH proton donors and amide C=O and phenyl ring proton acceptors which was evidenced by FTIR spectroscopy.[65] The C=O stretching vibration (amide I) band with a maximum at 1654-1640 cm⁻¹ and a shoulder at 1717 cm⁻¹, and the N-H bending vibration (amide II) band at 1544 cm⁻¹ characterized α -helical secondary conformation of polypeptide chain (**Fig. 4**).[66] The presence of β -sheet structures is illustrated here by the existence of a notable shoulder of amide I at 1620 cm⁻¹. [67–70] Alteration of the β -sheet structures by strong acids such as TFA, acting as proton donor, weaken the interchain peptidic hydrogen bonds, favoring solubilization. Although, the copolymers present structural similarity with UCST P(AAm-co-St) polymers, the prevalence of strong intermolecular hydrogen bonds and β -sheets supramolecular association prevents from obtaining a thermoresponsive behavior. Nonetheless, we were able to produce for the first-time copolypeptides of Asn-NCA and Phe-NCA by optimizing the polymerization conditions.

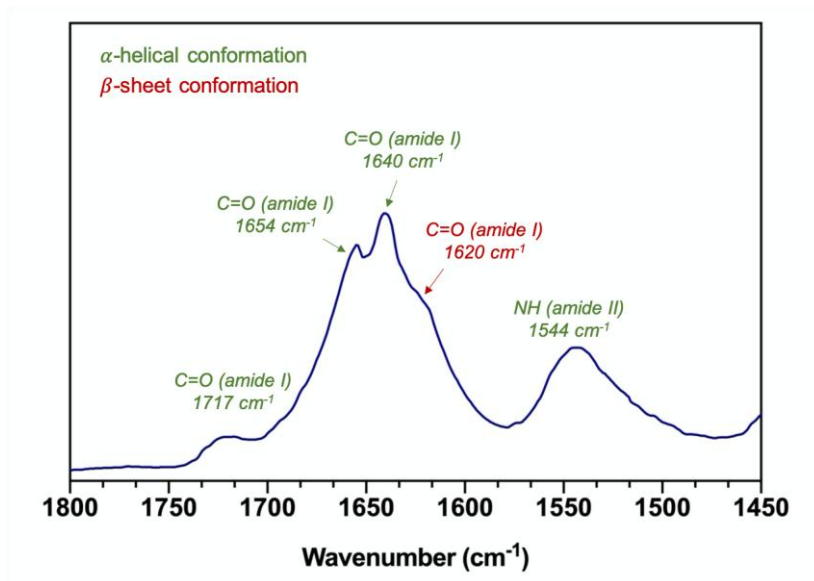


Fig. 4. FTIR of P(Asn-*co*-Phe) (ratio 90:10) obtained from the ROP of Asn-NCA and Phe-NCA in DMSO ($[M]/[I] = 50$) at 30°C (0.06M) in the 1800-1450 cm^{-1} region. The amide bands indicative for α -helical conformation of the peptide chain are assigned in green, the one indicative for β -sheet structures is marked in red.

Taking advantage of this naturally occurring peptide self-assembly, researchers have aimed at associating polypeptides to other biocompatible polymers, such as polyesters or polyethers to prepare well-defined nanoparticles for use in drug delivery applications.[71,72] In the light of our solubility assay, we aimed at accessing amphiphilic polypeptide-based nanoparticles *via* nanoprecipitation of designed PEGylated PAsn et PPhe polymers.

3.4. Preparation of PEG-*b*-PNCA diblock copolymers and nanoparticles

It has already been shown that hydrophobic interaction coupled with inter and intramolecular hydrogen bonding in polypeptides are driving forces for spontaneous self-assembly into micelles or vesicles regardless of the polymer molar masses.[63] These objects have been extensively used as drug delivery carriers in biomedical applications. For example, Akashi *et al.* reported the one-pot preparation of PEGylated PPhe nanospheres using dual initiators in a water/DMSO solvent mixture.[23] Driven by the biocompatibility and inert characteristics of

PEG, PEGylation is a frequently used technique for imparting “stealth” properties to drug delivery nanocarriers.[71] Based on the findings discussed above, the scope of our research was extended to the preparation of amphiphilic PEG-*b*-PAsn and PEG-*b*-PPhe copolymers nanoparticles. On one hand, Asn-NCA and Phe-NCA appeared monomers of choice for providing hydrogen bonds and hydrophobicity. On the other hand, a low molecular weight polyethylene oxide amine (PEG_{5k}-NH₂) was selected as PEG-based macroinitiator.

Two diblock copolymers were prepared by ROP of Asn-NCA or Phe-NCA from PEG_{5k}-NH₂ macroinitiator at a monomer-to-initiator ratio of 60 ([M]/[I] = 60) at 30°C in anhydrous DMSO (0.4 M). Chain growths of Asn-NCA and Phe-NCA from PEG_{5k}-NH₂ were first assessed by ¹H NMR spectroscopy, showing the resonances of both the methylene proton (-CH-) adjacent to the carbonyl of the carboxyanhydride that shifted downfield from $\delta = 4.50$ -4.53 (triplet) to 4.45 ppm (multiplet) and from $\delta = 4.51$ -4.55 (dd) to 4.52 ppm (multiplet), respectively, as a result of the formation of peptide bonds (**Fig. S20** and **Fig. S21** in ESI). ¹H NMR spectroscopy was again presumed to be the most accurate method to determine absolute polymer molecular weights not only due to solubility issues in most organic and aqueous solvents, including water, but also thanks to the high proton density of the PEG block allowing precise integration. The DP_n measured by ¹H NMR ethylene glycol groups analysis at $\delta = 3.47$ ppm, revealed that ≈ 20 Asn repeat units and ≈ 9 Phe were incorporated from the PEG_{5k}-NH₂ block to give the desired diblock copolymers with molar masses of $M_n = 7.8$ kg.mol⁻¹ and 6.8 kg.mol⁻¹, respectively. The lower number of Phe units incorporated from the PEG_{5k}-NH₂ block can be explained by the lower solubility of the polypeptidic block leading to early aggregation occurring within minutes during polymerization.

The ability of the PEG₁₂₅-*b*-PAsn₂₅ and PEG₁₂₅-*b*-PPhe₁₀ to form nanoparticles upon nanoprecipitation in aqueous medium was then assessed by DLS measurements (**Table 3** and **Fig. 5** and **Fig. S22** in ESI).

Table 3. Macromolecular and colloidal characteristics of NPs prepared by nanoprecipitation of PEG-*b*-PNCA diblock copolymers.

Entry	Polymer	$M_{n, NMR}^a$ (kg.mol ⁻¹)	D_z^b (nm)	PSD ^b	D_z^c (nm)	PSD ^c	ζ (mV) ^c
1	PEG- <i>b</i> -PAsn	7.8	273 ± 2.6	0.09 ± 0.02	338 ± 8.4	0.2 ± 0.02	4.7 ± 1.6
2	PEG- <i>b</i> -PPhe	6.8	760 ± 74.7	0.6 ± 0.04	- ^d	- ^d	- ^d

^a Determined by ¹H NMR. ^b Intensity-average diameter measured by dynamic light scattering (DLS) as an average of three different measurements before TFA removal. ^c Measured by dynamic light scattering (DLS) as an average of three different measurements after TFA removal. ^d Precipitation occurred.

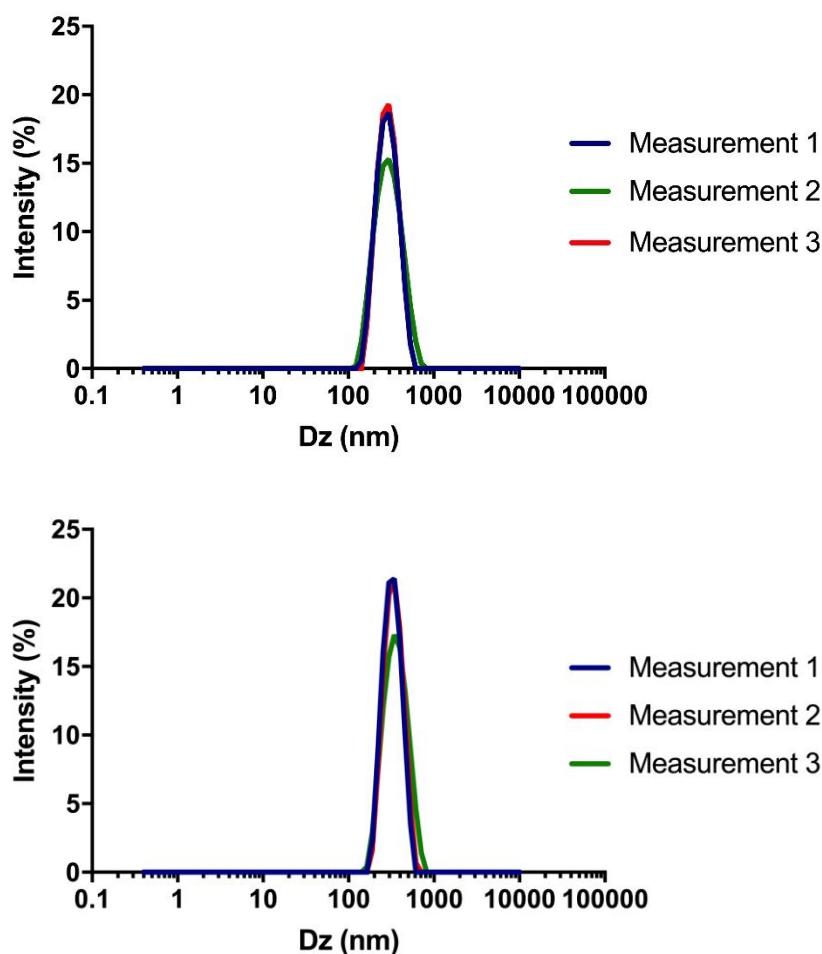


Fig. 5. Colloidal characteristics of NPs prepared by nanoprecipitation of PEG-*b*-PAsn diblock copolymers before TFA removal (top) and after TFA removal (bottom) as measured by DLS.

Overall, NPs differed greatly between PEG-*b*-PAsn and PEG-*b*-PPhe. While relatively low particle size of 273 nm was obtained with PEG-*b*-PAsn with narrow particle size distribution (PSD) < 0.1 (**Fig. 5**), large aggregates formed upon nanoprecipitation of PEG-*b*-PPhe ($D_z = 760$ nm, PSD = 0.6) (**Fig. S22** in ESI). After careful removal of TFA under reduce pressure, the stability of PEG-*b*-PAsn NPs proved slightly impacted with an increase in particle size from 272 up to 338 nm (PSD = 0.2) (**Fig. 5**). As for PEG-*b*-PPhe NPs, removal of TFA led to a tremendous increase in particle size – which in fact, was too large to be properly analyzed by DLS – and some precipitate could be observed, confirming the low colloidal stability of the particles. The ζ -potential of our PEG-*b*-PAsn NPs was of slightly positive value (~ 5 mV) which agrees with the tendency of PEGylated NPs to show rather near-neutral ζ -potential in

comparison with their non-PEGylated counterparts.[71] Providing further optimization of their colloidal features, these results showed that PEG-*b*-PAsn could be potentially used as nanocarriers by drug delivery applications.

Conclusion

The aim on this study was plural. First, a new optimized synthetic approach to pure L-phenylalanine and L-asparagine *N*-carboxyanhydrides (Phe-NCA and Asn-NCA, respectively) **in one-pot process has been developed based on two different carbonylation pathways**. Low solubility and aggregation phenomena caused by the numerous inter- and intramolecular hydrogen bonding interactions were minimized by setting proper polymerization conditions and as analogy with well-known poly(acrylamide-*co*-styrene) (P(Aam-*co*-St)) UCST copolymers, poly(asparagine-*co*-phenylalanine) (P(Asn-*co*-Phe)) copolypeptides of different Asn to Phe ratio were obtained. Thermoresponsivity of our P(Aam-*co*-St)-inspired polypeptides were assessed but polymer association *via* strong hydrophobic interactions favored by the presence of β -sheet structures that not only prevented the occurrence of a thermosensitive response in aqueous media but also precluded their solubilization in most organic solvents. Taking advantage of this naturally occurring self-assembly, PEGylated PAsn and PPhe polymers were prepared and nanoparticles formulation showed that PEG-*b*-PAsn could form NPs with relatively low particle size and narrow particle size distribution for potential application in drug delivery.

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Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

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Graphical abstract

