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Poly(amino acid) Synthesis from 2,5-Diketopiperazines for Acid-Actuated Drug Release

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Poly(amino acids) have enormous potential value as biomaterials owing to their inherent biocompatibility, chemical functionality, and biodegradability. However, current commercial poly(amino acid) use is somewhat limited due to production protocols that often include highly toxic phosgene in monomer synthesis. To circumvent this unfortunate predicament, the use of bio-renewable 2,5-diketopiperazines (2,5-DKPs) as cyclic monomers is proposed for poly(amino acid) synthesis. Amphiphilic block copolymers are produced by using poly(ethylene glycol) methyl ether (mPEG) as a macroinitiator, and pH-sensitive nanoparticles form capable of highly controlled, acid-actuated, doxorubicin release. This route to poly(amino acid) synthesis may facilitate the safe and economically viable use of key biodegradable polymers in both every day and high-value biomedical products, such as materials for stimuli-responsive drug delivery.

1. Introduction

2,5-Diketopiperazines (2,5-DKPs) are naturally abundant cyclic dipeptides in the form of conformationally constrained six membered heterocyclic rings. The cyclic structure contains two cis-amide linkages, ensuring that the nitrogen atoms and carbonyl groups occupy opposite sides of the ring, resulting in two H-bond acceptor sites and two H-bond donor sites.[1,2] 2,5-DKPs have found application as effective materials in numerous biomedical settings including as anti-microbial,[3] anti-cancer,[4] anti-inflammatory[5] and anti-fouling agents,[6] and as carriers for controlled payload release.[7] The potential to exploit 2,5-DKPs for material creation is significant owing to their unique and adaptable chemical properties and environmental credentials.

The controlled release of guest molecules from polymeric carriers may be exploited for the prolonged release of active molecules including fragrances, agrichemicals, and antioxidants. In particular, the encapsulation and controlled release of therapeutic molecules from polymer carriers remains a key challenge of medicine, healthcare materials, and fundamentally polymer science. Enabling the precise delivery of drug molecules in vivo is imperative as it protects the drug molecule from premature interaction with non-target healthy cells, resulting in often undesirable side-effects being felt by the patient in the absence of drug activity at the target site. Such premature drug activity results in increased drug dosage, which results in further detrimental side-effects and increased treatment costs. There has been enormous progress made in the development of polymeric drug delivery vehicles, however at a commercial scale drug delivery devices remain reliant on polyesters such as poly(lactic acid) to enable drug encapsulation within polymer nanoparticles.

Poly(amino acids) are particularly well suited for the creation of drug delivery vehicles as they are produced from a renewable feedstock (amino acids), are biocompatible, and are biodegradable by enzymatic hydrolysis, producing only water as a side product. Further to their environmental credentials, poly(amino acids) are polyamides that boast an extensive range of possible chemical functionalities based simply on the amino acid(s) that is/are polymerized. Such versatility in polymer design is uncommon in the synthesis of biodegradable polymers and allows polymers with different polarities, electrostatic charges, and pendant functional groups, including thiol, amine, carboxylic acid, and alcohol, to be created. As homo-polypeptides, extensive polymer-polymer interactions may form via hydrogen bonding, ensuring secondary structure formation that enables the creation of very stable (nano)structures including organogels,[8–10] hydrogels,[11–14] and enduring polymer nanoparticles,[15] for the encapsulation, and controlled release of guest therapeutics. Poly(amino acids) are therefore of enormous potential value, both as commodity polymers and as intricate components of biomaterials, with inclusion as components of drug delivery vehicles a clear potential use.[16–18] However, safe, sustainable, and

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cost-effective, methods for their synthesis must be found if such enormous potential is to be realized.

Although solid phase peptide synthesis and polycondensation reactions enable the creation of poly(amino acids),[19] the former requires multiple coupling steps, costly coupling agents, and the use of deprotection agents between coupling steps, which may involve the use of toxic reagents such as piperidine for Fmoc cleavage. Polycondensation reactions lack control and crucially do not permit the efficient production of block copolymers from AB_2 monomers such as amino acids. The monomers that form the second block may react together to form homopolymers without reacting with the macroinitiator that conventionally forms the first block, resulting in a mixture of homopolymers and block copolymers. Ring-opening polymerization circumnavigates this as second block ring-opening, and block copolymer propagation, is only achieved upon reaction with the active site that the first block macroinitiator provides, preventing reaction between cyclic monomers in the absence of the macroinitiator. Consequently, ring-opening polymerization is the most common method used for controlled poly(amino acid) creation. In particular, N-carboxyanhydride ring-opening polymerization (NCA ROP) is frequently used.[20] However, the commercial use of poly(amino acids) is limited owing to the economic and environmental cost of polymer synthesis via NCA ROP. Triphosgene is typically used for amino acid conversion to the corresponding NCA via the formation of highly-toxic phosgene,[21] which is lethal by inhalation. In addition, tetrahydrofuran (THF) is commonly used as the solvent for NCA synthesis, partially due to the NCA product, but not amino acid starting material, being soluble in THF. The recognized need for poly(amino acids), and the effectiveness of ROP over solid phase synthesis and polycondensation reactions has driven great interest into the development of phosgene-free synthetic routes to NCAs. Koga et al. reported the non-halogenated and much less toxic analogue of phosgene, diphenyl carbonate, to convert the imidazolium salt of an amino acid to the corresponding urethane, which can be cyclized in the presence of an acid catalyst.[22] Despite skillfully avoiding the use of phosgene, this procedure does require the use of the imidazolium salt rather than the free amino acid, as well as a minimum of 4 d to complete three synthetic steps and a fourth purification step, resulting in a lower yield when compared to the seemingly more straightforward, one-pot, Fuchs-Farthing method of NCA synthesis.[23] Other recent attempts that utilize similar synthetic steps are innovative but at present have comparable drawbacks,[24] ensuring that the use of phosgene in NCA creation for poly(amino acid) synthesis remains common. This yields a true scientific conundrum; poly(amino acids) are extremely useful polymers, but their creation from amino acid NCAs remains environmentally problematic and/or economically challenging due to use of cyclizing agents, such as phosgene, and volatile organic solvents created by harmful petrochemical production, such as THF,[25] in NCA creation.

As an alternative method to poly(amino acid) synthesis, we propose replacing the NCA with a 2,5-DKP as the cyclic monomer. 2,5-DKP synthesis via the condensation of two α-amino acids in ethylene glycol is an extremely straightforward, sustainable, and safe process. It involves one simple reaction step, minimal purification, and excludes both toxic organic solvents and phosgene-producing chemicals. 2,5-DKP ROP may then feasibly proceed from amino or hydroxyl initiators, with the latter enabling the creation of amphiphilic poly(ethylene glycol)-poly(amino acid) block copolymers.

Herein, the creation of poly(amino acids) from amino acid 2,5-DKPs is reported. Importantly, amphiphilic block copolymers are formed which cannot be created precisely in a straightforward manner by polycondensation, and toxic and costly reagents used for cyclic monomer synthesis are avoided. This method of poly(amino acid) synthesis potentially offers a safe, sustainable, and commercially-viable route to poly(amino acid) block copolymers, with suggested application including their use as controlled (drug) delivery vehicles, rheology modifiers, and biodegradable surfactants. The formation of pH-sensitive nanoparticles capable of encapsulating and releasing a chemotherapeutic payload on-command is reported, demonstrating the conceivable effectiveness of these materials as drug delivery vehicles.

2. Experimental Section

2.1. Materials and Methods

All chemicals used were purchased from Sigma-Aldrich and used as received unless otherwise stated. L-phenylalanine, 2,5-Piperazinedione ("Gly-DKP"), and diethyl ether were purchased from Fluorochem and used as received. L-alanine and ethylene glycol 99% were purchased from Alfa Aesar. Dimethylformamide (DMF) was purchased from ACROS chemicals. All chemicals were used without further purification.

2.2. General Synthesis of 2,5-Diketopiperazines

The general procedure for preparation of 2,5-DKPs is well-established.[1–3] Briefly, the amino acid (1 weight equivalent) was added to an oven-dried vessel and flushed with $N_{2(g)}$. The solid was then suspended in ethylene glycol (7 weight equivalents) and stirred at 170–190 °C under constant flow of $N_{2(g)}$. The reaction mixture was allowed to cool to room temperature, the precipitate collected by vacuum filtration, and washed at the filter with ice-cold methanol (30 mL). The solids were then recrystallized from hot deionized water (30 mL). The crystals were collected by vacuum filtration and dried in vacuo. Specific protocols for the particular 2,5-DKPs created can be found in the supporting information.

2.3. Synthesis of Poly(amino acid) Homopolymers

The respective 2,5-DKP and benzylamine were added to an oven-dried vessel and flushed with $N_{2(g)}$. For polymer entries in **Table 1**, stannous octoate ($Sn(Oct)_2$) was added and the mixture in anhydrous DMF and stirred at 130 °C under constant flow of $N_{2(g)}$. After 167 h, the reaction mixture was allowed to cool to room temperature and filtered by gravity. Ice-cold diethyl ether (90 mL) was added to aid polymer precipitation and the product collected by centrifuge (4500 rpm, 0 °C, 3 min). The solids were washed further with additional ice-cold diethyl ether (3×30 mL) and dried

Table 1. Details of the reagents used in polymer synthesis, and the composition of the poly(amino acids) formed.

	Initiator		Monomer		Solvent	Catalyst	Reaction Time [h]	Poly(amino acid) repeat units			Polymer formula
	Name	Mols [mmol]	Name	Mols [mmol]		Mols [mmol]		Target	Observed (NMR)	Observed (MALDI)	
1	Benzylamine	0.24	Gly-DKP	9.65	DMF	0.12	167	80	61	54	$PGly_{54}$
2	Benzylamine	0.089	Ala-DKP	3.52	DMF	0.045	167	80	64	68	$PAla_{68}$

in a vacuum oven (50 °C) overnight. For polyalanine (PAla) produced in deionized (DI) water from recovered Ala, benzylamine (1.5 mg, 0.014 mmol) was used as the initiator and diisopropylethylamine (DIPEA, 9.0 mg, 0.07 mmol)) and methanesulfonic acid (MSA, 0.0375 mmol) used as catalysts. Ala-DKP (99.5 mg, 0.7 mmol), benzylamine, and MSA were stirred in deionized water (10 mL) at 80 °C under constant flow of $N_{2(g)}$ for 8 h, at which point DIPEA was injected. After 167 h, the mixture was allowed to cool, after which it was dialyzed against deionized water for two days. The remaining contents within the dialysis tubing were frozen using liquid nitrogen and lyophilized.

2.4. Enzymatic Degradation of Polyalanine

PAla (Table 1, entry 2) (300 mg, 0.057 mmol) was suspended in DI water (10 mL). Elastase was added (8.28 enzymatic units) and the mixture was stirred at 37 °C for one week, after which the mixture was dialyzed (2000 Da molecular weight cut-off (M.W.C.O.) against DI water (100 mL) for a further 3 d with frequent water changes. The dialysate was frozen using liquid nitrogen and lyophilized.

2.5. Synthesis of Poly(ethylene glycol)-*b*-Poly(amino acid) Diblock Copolymers

The respective 2,5-DKP, poly(ethylene glycol) methyl ether (mPEG, average M_w = 5000 g mol^{-1}) and $Sn(Oct)_2$ were added to an oven-dried vessel and flushed with $N_{2(g)}$. The mixture was suspended in anhydrous DMF and stirred at 130 °C under constant flow of $N_{2(g)}$. After an allotted period of time (**Table 2**), the reaction mixture was allowed to cool to room temperature and filtered by gravity. The filtrate was precipitated by ice-cold diethyl ether (150 mL) and the solids collected by centrifugation (4500 rpm, 0 °C, 3 min).

For the polymerizations of glycine DKP and alanine DKP (where R = H and —CH_3 respectively), the solids were redissolved in minimal THF and the mixture centrifuged (4500 rpm, 0 °C, 3 min) to separate unreacted DKP which remained undissolved. The supernatant was then precipitated in ice-cold diethyl ether (3×30 mL) and dried in a vacuum oven (50 °C) overnight.

For polymerizations of phenylalanine DKP (where R = benzyl), the solids were suspended in toluene (30 mL) to dissolve unreacted phenylalanine DKP. The suspension was then centrifuged (4500 rpm, 0 °C, 3 min) and the supernatant decanted. The remaining solid was washed with ice-cold diethyl ether (3×30 mL) and dried in a vacuum oven (50 °C) overnight.

2.6. Preparation of PEG-*b*-Poly(amino acid) Copolymer Nanoparticles

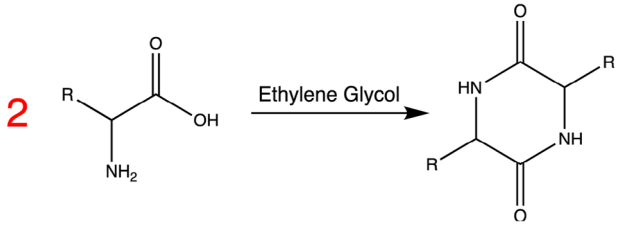
To create PEG-*b*-poly(amino acid) copolymer nanoparticles, each diblock copolymer (10 mg) was dissolved in chloroform (1 mL) and the solution added dropwise to DI water (10 mL) under vigorous stirring. The dispersions were stirred overnight and examined to ensure there was no visible organic phase.

2.7. Preparation of Doxorubicin (Dox) Free Base

Dox hydrochloride (2.00 mg, 3.67 µmol) was added to a solution of triethylamine (20 µL, 55 µmol) in anhydrous chloroform (3 mL). The solution was isolated from light and stirred at room temperature for 4 h.

Table 2. Details of polymer synthesis, and the composition of the poly(amino acids) formed for nanoparticle formation.

	Initiator		Monomer		Catalyst	Solvent vol. [mL]	Reaction time [h]	Poly(amino acid) repeat units			Polymeric formula
	Name	Mols [mmol]	Name	Mols [mmol]	Mols [mmol]			Target	Achieved (NMR)	Achieved (APC)	
3	$mPEG\text{-}OH_{5000}$	0.053	Gly-DKP	2.63	0.46	40	162	100	31	29	PEG_{112}-*b*-$PGly_{31}$
4	$mPEG\text{-}OH_{5000}$	0.1	Gly-DKP	0.7	0.46	20	239	14	14	13	PEG_{112}-*b*-$PGly_{14}$
5	$mPEG\text{-}OH_{5000}$	0.08	Gly-DKP	0.8	0.04	20	332	20	20	19	PEG_{112}-*b*-$PGly_{20}$
6	$mPEG\text{-}OH_{5000}$	0.035	Ala-DKP	2.11	0.018	10	118	20	6	6	PEG_{112}-*b*-$PAla_{6}$
7	$mPEG\text{-}OH_{5000}$	0.053	Ala-DKP	2.11	0.027	10	118	80	14	11	PEG_{112}-*b*-$PAla_{14}$
8	$mPEG\text{-}OH_{5000}$	0.08	Phe-DKP	1.2	0.04	40	120	30	25	24	PEG_{112}-*b*-$PPhe_{25}$



Scheme 1. Direct amino acid condensation to form a 2,5-diketopiperazine, for Ala, R = CH_3 for phenylalanine (Phe), R = phenyl.

2.8. Preparation of Dox-Loaded Nanoparticles

The polymers (10 mg) were independently dissolved in chloroform (1 mL). Dox was then encapsulated within the nanoparticles that formed from each respective polymer as follows: the respective polymer solutions and Dox-free base solution (2 mg mL^{-1}, 3.67 µmol) were added simultaneously, in a dropwise fashion, to vigorously stirring buffer solution (PBS, pH 7.4, 10 mL). The nanoparticles were dialyzed against deionized water (2000 Da M.W.C.O.) for one week with frequent water changes to remove excess free doxorubicin.

2.9. pH-Mediated Release of Dox from Dox-Loaded Nanoparticles

The Dox-loaded nanoparticle solutions were split into two equal portions by volume and decanted into fresh dialysis tubing (2000 Da M.W.C.O.). One Dox-loaded nanoparticle dispersion was then dialyzed (M.W.C.O. 2000 Da) against an acetate buffer (pH 5.0) solution, while the other was dialyzed (M.W.C.O. 2000 Da) against fresh PBS buffer solution (pH 7.4). The vessels were incubated in the dark under constant agitation at 37 °C. At predetermined intervals, 1 mL aliquots were removed from the dialysate and analyzed by UV–vis spectrophotometry. The amount of dox released at each time point was quantified by UV-vis spectrophotometry using a prepared standard calibration curve (Figure S46, Supporting Information). The release from the nanoparticles was studied over the course of 194 h.

3. Results and Discussion

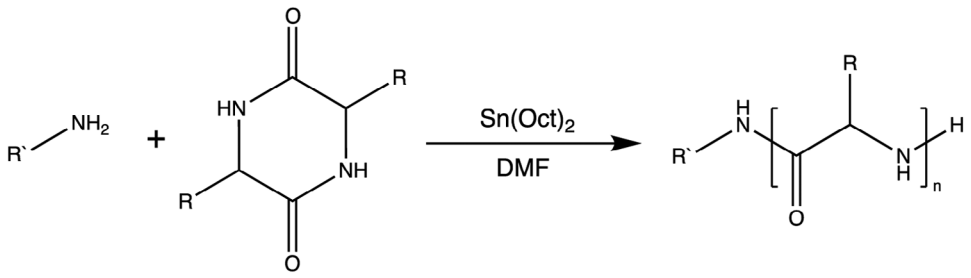
3.1. 2,5-Diketopiperizine Synthesis

This research aimed to determine if poly(amino acids) can be created from 2,5-DKPs, enabling a new route to both homopolymers and block copolymers that may be applied as drug delivery vehicles. Initially, polymers containing Ala and Gly repeat units were synthesized owing to the reported biodegradation of the corresponding poly(amino acids) by elastase,[26] which may be exploited as a trigger for drug release, or enable monomer recovery via polymer hydrolysis. In addition, both DKP monomers are water-soluble at the polymerization temperature, and extensive hydrogen bonding between PAla and PGly ensures that polymers can be created to high mechanical strength.[27] Extensive hydrogen bonding between polymer chains may also be exploited to drive very stable nanoparticle formation. Ethylene glycol was selected as the solvent for DKP synthesis owing to its minimal toxicity and relatively high boiling point (197.3 °C). 2,5-DKPs form via the direct bis-condensation of two amino acid molecules where the amine group of one amino acid nucleophilically attacks the carbonyl group of the second amino acid, and vice versa (**Scheme 1**). Ala-DKP was obtained as off-white crystals and their successful syntheses confirmed by FTIR spectroscopy (Figure S1, Supporting Information), ^{1}H NMR spectroscopy (Figure S3, Supporting Information), and LC-MS (Figure S5, Supporting Information). Gly-DKP can be made in an analogous manner, but the commercial product was used in this research. This facile method of 2,5-DKP synthesis enables the generation of a wide-range of potential monomers in the absence of other reagents.

3.2. Homo-Poly(amino acid) Synthesis

2,5-DKPs are six membered rings that do not possess the ring-strain of five-membered NCAs, rendering polymerization less favorable. However, the ROP of δ-valerolactam (2-piperidinone) to yield nylon 5 has been reported,[28] and so the absence of 2,5-DKP ROP from the literature is somewhat surprising. Initially, each monomer was polymerized using a primary amine-presenting initiator and $Sn(Oct)_2$ as an FDA-approved catalyst (**Scheme 2**).[29] The optimal rate of ROP using $Sn(Oct)_2$ as a catalyst in ROP has been reported to be 130 °C,[30] with heat required due to the stability of the 6-membered 2,5-DKP ring. Although disadvantageous for polymerization, such monomer stability makes 2,5-DKP storage at room temperature significantly more straightforward than NCA storage, which often requires an inert atmosphere at –18 °C to prevent premature ROP. Also, DKP ROP proceeds without the loss of CO_2 upon each monomer addition to the polymer chain, demonstrating excellent atom economy.

It may be hypothesized that polymerization proceeds due to the strong affinity that $Sn(Oct)_2$ has for oxygen-containing functional groups, such as the amide groups of the DKP, which



Scheme 2. 2,5-DKP ROP initiated by benzylamine when R' = benzyl, and hexylamine when R' = hexyl. The polymerization is catalyzed by $Sn(Oct)_2$.

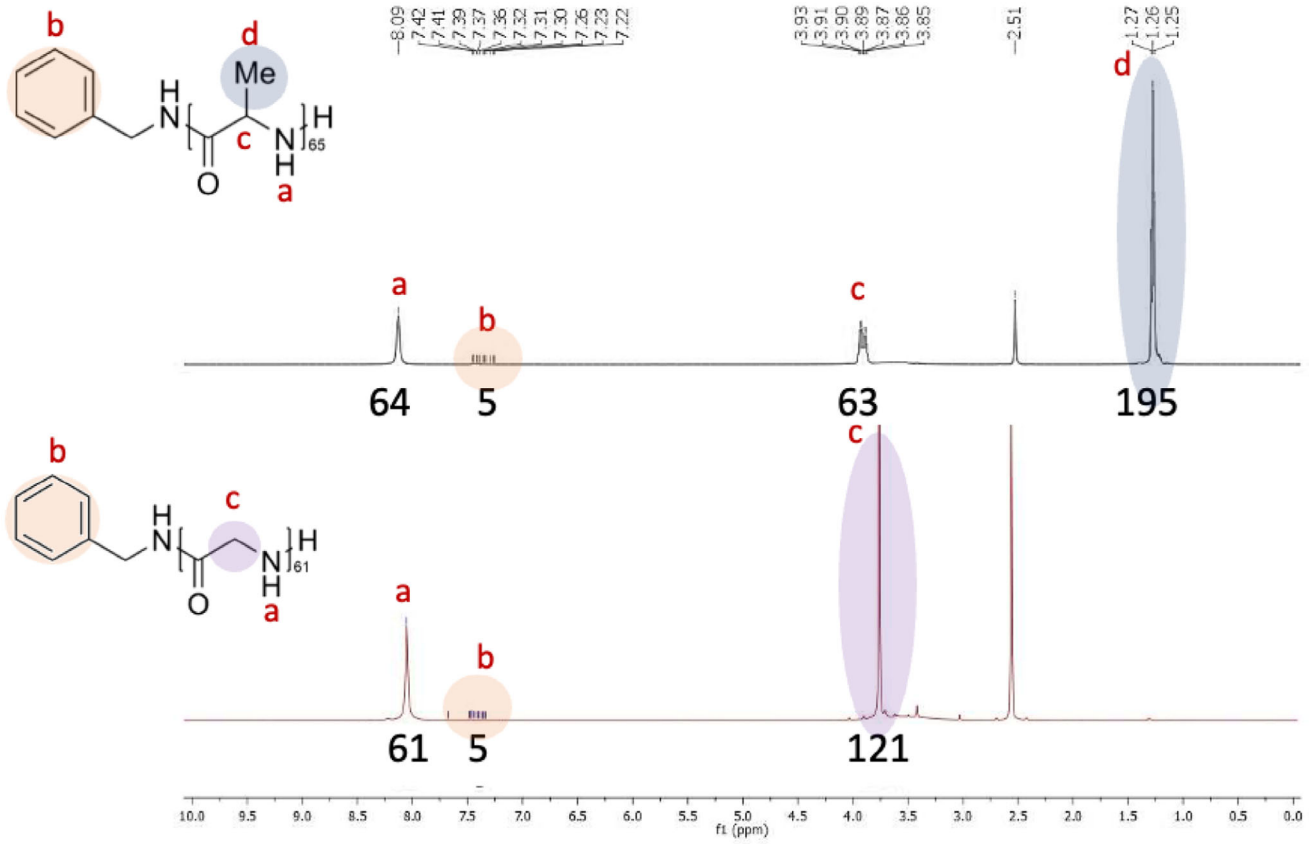


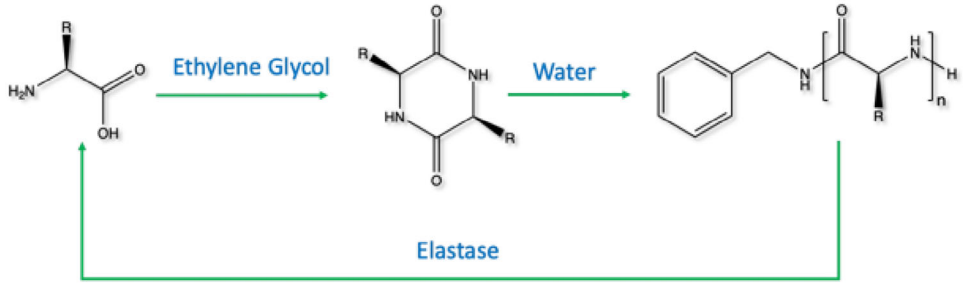
Figure 1. ^{1}H NMR spectra $PAla_{64}$ (top), and $PGly_{61}$ (bottom). The number in blue font beneath each peak signifies the integral intensity of the peak normalized against the aromatic protons of the initiator. All spectra were recorded at 500 MHz using a mixture of trifluoroacetic acid-d (TFA-d) and DMSO-d6 (peak at 2.5 ppm) (TFA-d/DMSO-d6).

enables catalyst coordination. Such coordination to the carbonyl oxygen of the amide groups increases the electrophilicity of the carbonyl cation, which sufficiently weakens the C-N bond of the amide, rendering it susceptible to nucleophilic attack by initiating or propagating amine groups.

PGly and PAla were characterized using FTIR spectroscopy (Figures S7 and S8, Supporting Information), ^{1}H NMR spectroscopy (Figures S9 and S10, Supporting Information), and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Figures S11 and S12, Supporting Information). MALDI-TOF mass spectrometry was selected for analysis due to the insolubility of PAla and PGly in typical GPC solvents (THF and DMF) at working temperatures (40 °C) following their recovery and purification. MALDI-TOF mass spectrometry provides a direct mass distribution, as opposed to mass analysis based on polymer hydrodynamic volume that may be calibrated against molecularly dissimilar standards. It should be noted that although PAla and PGly appeared to remain in solution during polymerization at 130 °C, following their recovery these polymers could not be re-dissolved in DMF. The lack of solubility of PAla and PGly in both DMF and water is a key indicator for successful 2,5-DKP ROP, at least to some extent. Following synthesis, all polymers were dialyzed against DI water for 48 h with frequent water changes to remove unreacted and water-soluble Ala-DKP or Gly-DKP.

PGly (Table 1, entry 1), revealed a peak at 3.94 ppm in the ^{1}H NMR spectrum (**Figure 1**) corresponding to the CH_2 of the PGly repeat unit. The integrals of the spectrum were normalized to the five protons of benzylamine which gave the CH_2 peak an integration value of 122, signifying 61 repeat units and 76% monomer conversion. Analysis by MALDI-TOF mass spectrometry gave a distribution with number averaged molecular weight (M_n) = 2900 g mol^{-1} and weight averaged molecular weight (M_w) = 3100 g mol^{-1}, signifying 54 repeat units and a narrow dispersity of 1.06. This showed agreement with the ^{1}H NMR spectroscopic analysis, and provides evidence for poly(amino acid) synthesis.

PAla creation by NCA ROP can be problematic owing to difficulties in alanine NCA synthesis that result in limited NCA yields.[31] We have also observed undesirable premature ROP of Ala NCA in the absence of an initiator owing to the susceptibility of the NCA to undergo hydrolysis. The ^{1}H NMR spectrum of PAla revealed three peaks (Figure 1); 1.26 ppm corresponding to the CH_3, 3.84-3.93 ppm corresponding to the CH, and 8.08 ppm corresponding to the NH of the repeat unit. The integration values normalized to the aromatic protons of benzylamine gave an average degree of polymerization of 64 repeat units and 80% monomer conversion. Analysis by MALDI-TOF mass spectrometry gave a distribution with Mn = 4900 g mol^{-1} and Mw = 5300 g mol^{-1}, signifying 71 repeat units and a dispersity of 1.07, again showing reasonable agreement with ^{1}H NMR



Scheme 3. Enzymatic degradation of PAla via incubation with elastase at 37 °C, followed by the cyclization of the Ala obtained to create Ala-DKP, and ROP of Ala-DKP to synthesize PAla.

analysis. Creating PAla, a polymer reported to self-assemble and form both α-helix and β-sheet secondary structures and be a component of spider silk, in such a straightforward, unproblematic, manner is notable for the production of advanced biomedical and engineering materials, particularly the creation of stable nanoparticles for drug delivery.[32–34]

3.3. PAla Circularity via DKP ROP

A key target in polymer science is the production of useful polymers that can be recovered, depolymerized, and the obtained monomers reused. Poly(amino acids) may be designed to be susceptible to enzyme-catalyzed hydrolysis, yielding the amino acid starting material and water as natural products.[35] Enzyme-mediated hydrolysis ensures that enzyme-responsive materials may be used in biomedical applications, for instance for the highly selective release of drug molecules triggered by a specific target enzyme.[36] In addition, polymer degradation in response to a target enzyme may allow amino acid recovery, DKP reformation, and poly(amino acid) synthesis (**Scheme 3**) in water. The water solubility of Ala-DKP at the polymerization temperature renders polymerization in this solvent feasible.

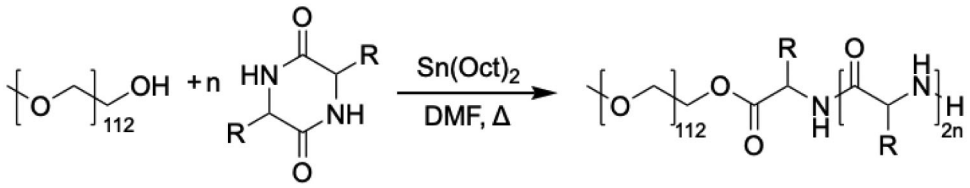
The elastase-mediated biodegradation of PAla ($PAla_{64}$, Table 1, entry 2) was assessed, with the polymer being incubated in enzymatic solution at 37 °C for one week. The degradation products were analyzed by ^{1}H NMR spectroscopy (Figure S11, Supporting Information) and HPLC (Figure S13, Supporting Information), with complete degradation of polymer to Ala apparent. 300 mg of $PAla_{64}$ yielded 240 mg of Ala, representing close to 100% monomer recovery, with the remaining mass lost as water. The recovered Ala was then cyclized to 113 mg of Ala-DKP according to the procedure described, and the product characterized by FTIR spectroscopy (Figure S14, Supporting Information) ^{1}H NMR spectroscopy (Figure S15, Supporting Information), and LC-MS (Figure S16, Supporting Information). DKP ROP in water yielded $PAla_{45}$ (^{1}H NMR spectroscopy, Figure S18, Supporting Information) or $PAla_{39}$ (MALDI TOP mass spectrometry, Figure S19, Supporting Information) at 78% monomer conversion, demonstrating effective 2,5-DKP monomer creation and subsequent repolymerization of amino acids obtained enzymatically from an existing poly(amino acid). Such circularity was enabled by the ease in which amino acids may be obtained by enzymatic hydrolysis, and then readily re-converted to monomers (2,5-DKPs) in the absence of any toxic reactants or organic solvents. Importantly, this also shows that DKP ROP can be performed successfully in water. In the case of PAla synthesis, this is undoubtedly aided by the water-solubility of Ala-DKP, which contrasts to Ala-NCA.

The polymerization of Gly DKP and Phe DKP were then attempted in DI water using benzylamine as the initiator, targeting 80 repeat units in both cases. $PGly_{52}$ was produced (Figure S20, Supporting Information) demonstrating the effectiveness of DKP ROP for water-soluble monomers (Gly DKP). However, Ala and Gly aside, the vast majority of amino acid DKPs are water-insoluble, even at elevated temperatures. Phe DKP could undergo ROP in DI water in a heterogeneous system, but the polymer chain length was limited to 18 repeat units ($PPhe_{18}$) (Figure S21, Supporting Information), suggesting less polar solvents are essential for efficient DKP ROP of amino acid DKPs that are water insoluble.

3.4. pH-Responsive Polymer Nanoparticles Created from 2,5-DKPs

Poly(amino acids) can form very stable nanoparticles that prevent payload leakage owing to the hydrogen bonds between polymer chains that maintain the particle morphology. In the context of oncology, encapsulating cytotoxic chemotherapeutic molecules within polymer nanoparticles improves the pharmacokinetic profile of the therapeutic, enabling an increased drug concentration to reach the target site, reducing the development or intensity of side effects.[37] Drug delivery systems have been reported for the release of anti-cancer drugs in response to polymer hydrolysis,[38] for instance due to acidic conditions,[39] the presence of target enzymes capable of actuating polymer hydrolysis.[40] Creating stimuli-responsive drug delivery vehicles from bio-renewable monomers created by a straightforward synthetic protocol that avoids toxic and costly reagents remains a key target within this field.

Poly(ethylene glycol) (PEG) acts as a convenient hydrophilic macroinitiator for the production of amphiphilic block copolymers that contain poly(amino acid) segments as the hydrophobic block. PEG-*b*-poly(amino acid) block copolymers have shown great promise for the creation of polymeric nanoparticles, particularly if extensive intermolecular interactions exist between adjacent poly(amino acid) chains.[15] Poly(amino acids) formed from the 2,5-DKPs of Gly, Ala, and phenylalanine (Phe) are logical choices owing to hydrogen bonding that is not disrupted



Scheme 4. ROP of a DKP, initiated by mPEG (Average M_w = 5000 g mol^{-1}) and catalyzed using Sn(Oct)$_2$.

by the amino acid side-groups (Ala and Gly), and the potential for π–π interactions between the aromatic groups of Phe. Such intermolecular forces aid nanoparticle formation and enable prolonged nanoparticle stability in aqueous solution. PEG methyl ether (mPEG) was used as the macroinitiator to ensure the formation of diblock copolymers that contain an ester link forms between the mPEG and poly(amino acid) blocks. This ester link offers pH-sensitivity to the nanoparticles that may be exploited as an actuator for acid-catalyzed hydrolysis, and subsequent nanoparticle disassembly in acidic solution.[15]

The inclusion of mPEG within the amphiphilic block copolymers enables their dissolution in DMF following recovery. Consequently, size-exclusion chromatography analysis by advanced polymer chromatography (APC, analogous to gel permeation chromatography (GPC)) could be performed, providing further evidence for 2,5-DKP ROP.

Three classes of amphiphilic PEG-*b*-poly(amino acid) block copolymers were created by DKP ROP, using mPEG (M.W. 5000 g mol^{-1}) as the hydrophilic macroinitiator (**Scheme 4**, Table 2). The PEG-*b*-PGly block copolymers formed (Table 2, polymers 3, 4, and 5) were characterized using FTIR spectroscopy (Figures S22–S24, Supporting Information) and ^{1}H NMR spectroscopy (Figures S25–S27, Supporting Information). Both the CH_2 of PGly and the CH_2 protons of mPEG come to resonance at ≈3.65 ppm in ^{1}H NMR spectra. By comparing the integration of this peak in the ^{1}H NMR spectrum of unreacted mPEG to that of the peak in the product polymer spectra, the integration of the CH_2 peak in the PGly block of the product, and consequently the number of repeat units of the PGly block and monomer conversion, can be obtained.

After calibrating the integrals of the peaks in the spectrum to the reference peak, the main peak of mPEG at ≈3.65 ppm has an integral of 450. This agrees with the number of protons expected in 112 repeat units of mPEG with a molecular weight of 5000 g mol^{-1}. Using this methodology, the integration of the peak corresponding to the PGly block of polymer 3 was found to be 62, signifying 31 repeat units and 31% monomer conversion. APC reported the average M_n to be 6500 g mol^{-1}, the average M_w to be 6800 g mol^{-1}, and the block copolymer polymer dispersity to be 1.04 (**Figure 2**), showing good agreement with the ^{1}H NMR spectrum. Polymers 4 and 5 (Table 2) were characterized in the same fashion, revealing 14 and 20 repeat units of the PGly block, respectively. APC data showed good agreement with the values determined by ^{1}H NMR spectroscopy, giving average M_n of 5600 g mol^{-1}, average M_w of 5800 g mol^{-1}, and a narrow dispersity of 1.04 for polymer 6, and average M_n of 6100 g mol^{-1} and average M_w of 6200 g mol^{-1} with a narrow very dispersity of 1.02 for polymer 7 (Figure 2).

PEG-*b*-PAla block copolymers were then synthesized via the ROP of Ala-DKP from mPEG (M.W. 5000 g mol^{-1}) (Table 2, entries 6 and 7). Both polymers were characterized using FTIR spectroscopy (Figures S28 and S29, Supporting Information) and ^{1}H NMR spectroscopy (**Figure 3**). The CH_3 of PAla comes to resonance in a ^{1}H NMR spectrum at ≈1.9 ppm, whereas the CH of PAla comes to resonance at 4.14–4.23 ppm. Accurate integration of these peaks can be obtained by comparison to that of the reference peak (terminal CH_3 of mPEG) at ≈3.33 ppm. Integrals of the peaks at 1.45–1.50 ppm and 4.14–4.23 ppm suggested that polymers 6 and 7 contained 6 and 14 repeat units Ala units, respectively, which was comparable to APC data (**Figure 4**). Although the extent of monomer conversion is disappointing in both cases (30% and 17.5%, respectively), PAla blocks of limited repeat units may form very stable nanoparticles in aqueous solution due to extensive hydrogen bonding between polymer chains. Additionally, exceedingly lengthy PAla chains may render the block copolymer insoluble in non-fluorinated organic solvents, and lead to extensive and undesirable aggregation in aqueous solutions when the creation of nanoparticles is attempted.

Finally, Phe-containing block copolymers were created in the same manner. Phe-DKP was produced in an analogous manner to Ala-DKP synthesis (Supporting Information). Polymer 8 (Table 2) was characterized using FTIR spectroscopy (Figure S33, Supporting Information) and ^{1}H NMR spectroscopy (Figure S33, Supporting Information). By calibrating the Phe phenyl integrals

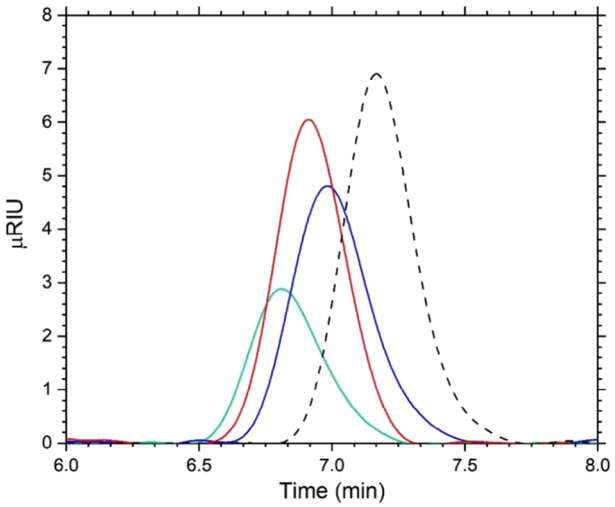


Figure 2. APC chromatograms of mPEG$_{5000}$-OH (black, dashed), and the deblock copolymers PEG$_{5000}$-*b*-PGly$_{31}$ (green), PEG$_{5000}$-*b*-PGly$_{14}$ (blue), and PEG$_{5000}$-*b*-PGly$_{20}$ (red).

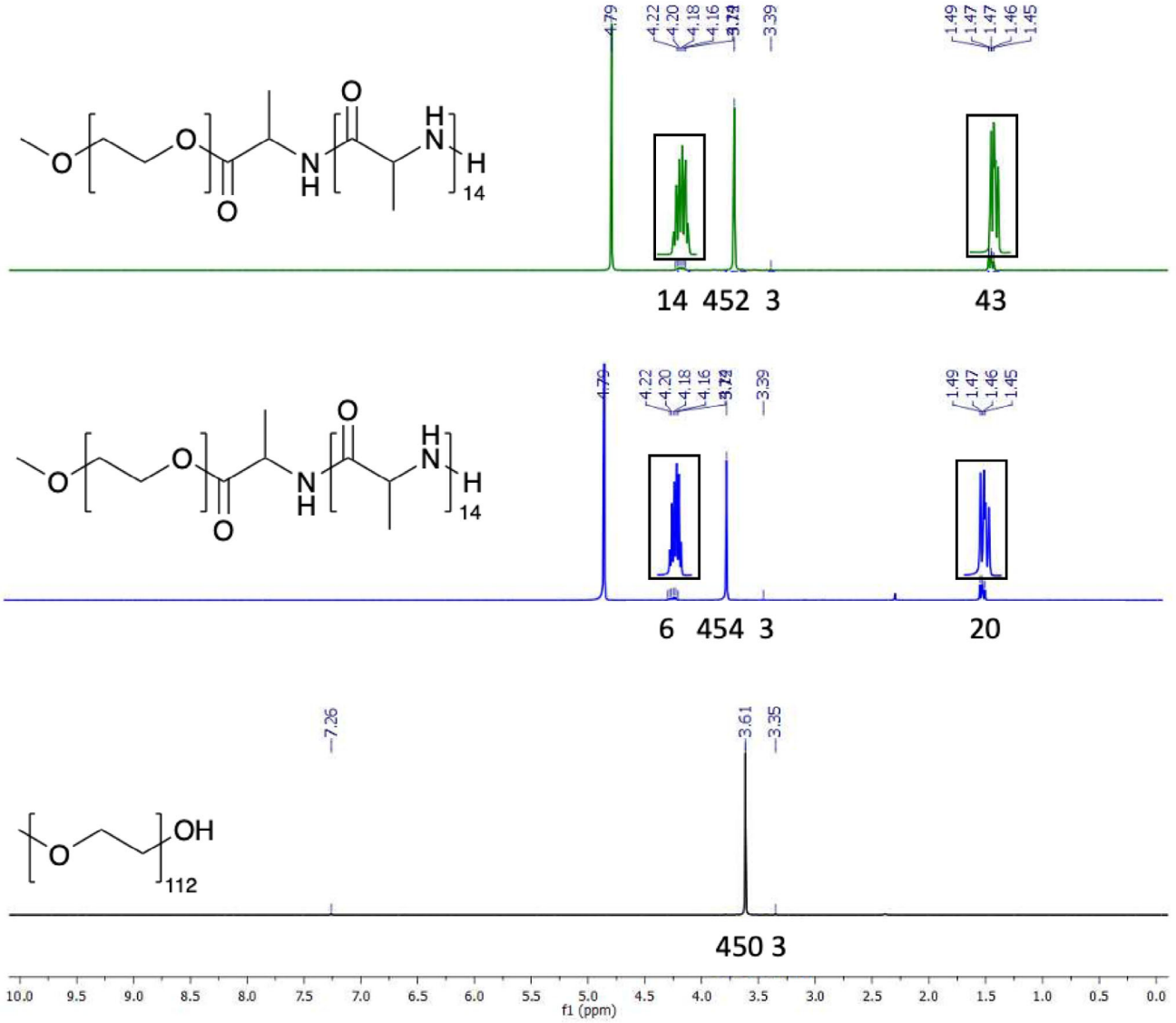


Figure 3. ^{1}H NMR of mPEG (M.W. 5000 g mol^{-1}) (black), polymer 6, PEG$_{5000}$-*b*-PAla$_{6}$ (blue) and polymer 5 = 7, PEG$_{5000}$-*b*-PAla$_{14}$ (green). 500 MHz, TFA-d/DMSO-d6.

of the ^{1}H NMR peaks to the terminal methyl peak of mPEG, the number of repeat units of the PPhe block was found to be 25. APC revealed the average M_n to be 8300 g mol^{-1} and the average to be M_w 8600 g mol^{-1}, with a narrow dispersity of 1.05 (**Figure 5**).

Table 3 provides a critical summary of PAla creation via polycondensation, NCA ROP, and DKP ROP. The three synthetic methods have benefits and drawbacks in both monomer and polymer synthesis; the main limitation of DKP ROP being the requirement of a catalyst and high temperature in the polymerization stage.

Nanoparticle formation was then attempted from the block copolymers produced, with DLS analysis performed. All six block copolymers were capable of self-assembly to form broadly spherical nanoparticles in aqueous solution at 1 mg mL^{-1} polymer concentration (Figures S34 and S35, Supporting Information and Table 3). In all cases the PDI value of the nanoparticles formed was less than 0.3, suggesting moderate monodispersity. Based on their dimensions, nanoparticles of polymer 6 may be applicable for intravenous/intramuscular administration, while nanoparticles of polymers 3, 4, 5 and 7 have the potential for ocular application.[41] Due to having an average particle size of less than 100 nm, nanoparticles of polymers 5 and 8 may have the capacity to cross the blood-brain barrier and consequently applicable as a therapeutic for brain tumors.[42] The nanoparticles were stored in aqueous solution for 6 months with no appreciable differences in particle size or dispersity found. Additionally, although the nanoparticles tested were created at 1 mg mL^{-1}, stable nanoparticle formation is possible at 0.1 mg mL^{-1} (e.g., Polymer 7: size = 186 nm, dispersity = 0.29) (**Table 4**).

Following these results, nanoparticles formed from polymers 4, 5, 6, and 7 were assessed for their capacity to encapsulate Dox free-base, and subsequently release the chemotherapeutic

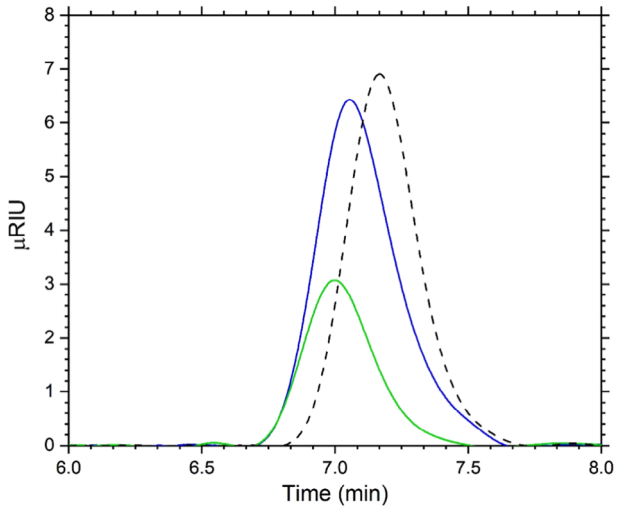


Figure 4. APC chromatogram of $mPEG_{5000}$-OH (black, dashed), PEG_{5000}-*b*-$PAla_6$ (blue), and PEG_{5000}-*b*-$PAla_{11}$ (green).

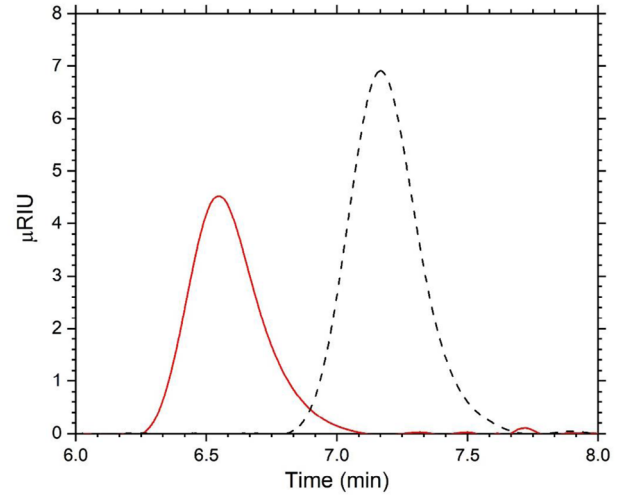


Figure 5. APC chromatogram of $mPEG_{5000}$-OH (black, dashed) and PEG_{5000}-*b*-$PPhe_{25}$ (red).

Table 4. The mean particle size and dispersity values of polymers 3–8 as determined by DLS and SEM analyses.

	Formula	DLS measurements		SEM measurements	
		Particle size (d.nm)	Dispersity	Particle size (d.nm)	Dispersity
3	PEG_{112}-*b*-$PGly_{31}$	210	0.22	180	0.24
4	PEG_{112}-*b*-$PGly_{14}$	180	0.20	190	0.19
5	PEG_{112}-*b*-$PGly_{20}$	90	0.17	100	0.22
6	PEG_{112}-*b*-$PAla_6$	190	0.19	210	0.23
7	PEG_{112}-*b*-$PAla_{14}$	180	0.24	190	0.17
8	PEG_{112}-*b*-$PPhe_{25}$	70	0.13	80	0.18

under acidic conditions that are associated with the endosomes of cells. When compared to extracellular pH of healthy tissues and blood at pH 7.4, the extracellular pH of most solid tumors is lower, ranging from pH 6.5 to 7.2 owing to the Warburg effect. Within cancerous cells the lysosomal pH is between 4.0 to 5.0. The enhanced permeation and retention (EPR) effect offers a mechanism for nanoparticles to accumulate at the tumor site whereby acid-catalyzed polymer hydrolysis may cause nanoparticle disruption and chemotherapeutic release.[43] Each polymer synthesized for drug release studies contains an ester link between the PEG block and the poly(amino acid) block, which is cleavable by acid-catalyzed hydrolysis. Therefore, acidic conditions serve as an appropriate trigger for the evaluation of these nanoparticle dispersions as potential drug delivery vehicles for anti-cancer therapy.

Dox encapsulation for polymers 4, 5, 6, and 7 was achieved at 92 ± 3 wt%, 95 ± 1 wt%, 87 ± 6 wt%, and 92 ± 5 wt% respectively, with corresponding drug loading contents of 15.5 ± 0.5 wt%, 16.0 ± 0.1 wt%, 14.8 ± 0.9 wt% and 26.9 ± 1.1 wt%. The release of Dox from the loaded nanoparticles was then assessed against aqueous solutions of pH 5.0 and pH 7.4, with the latter used to simulate physiological fluid. A temperature of 37.0 °C was maintained to further simulate physiological conditions. There was a profound difference in the extent of Dox release to pH 5.0 solution compared to release to pH 7.4 solution in all cases (**Figure 6**). Rapid "burst" release of Dox was avoided and crucially, Dox release to pH 7.4 solution was negligible in all cases up to

Table 3. A summary of the common routes to polyalanine.

Consideration	Polycondensation	NCA ROP	DKP ROP
Monomer synthesis (reactants)	No monomer synthesis	Challenging—cyclizing agents and scavengers required.	Straightforward—no cyclizing agents required.
Monomer synthesis (solvent)	No monomer synthesis	Organic solvents such as THF are commonly used.	Can be performed in aqueous solution.
Monomer purification	No monomer synthesis	Extensive—unreacted cyclizing agents and side products must be removed.	Straightforward—water is produced as the only side-product.
Polymerization	Uncontrolled. Can be done at room temperature.	Very controlled, no catalyst required. Organic solvents such as DMF required.	Straightforward but less controlled than NCA ROP. Catalyst and high temperature required.
Atom economy	H_2O loss during polymerization.	HCl loss during NCA formation. CO_2 loss during NCA ROP.	H_2O loss during DKP synthesis. No atom loss during polymerization.
mPEG-initiated block copolymer synthesis	Not readily possible.	Straightforward.	Possible, although reduced monomer conversion compared to NCA ROP.

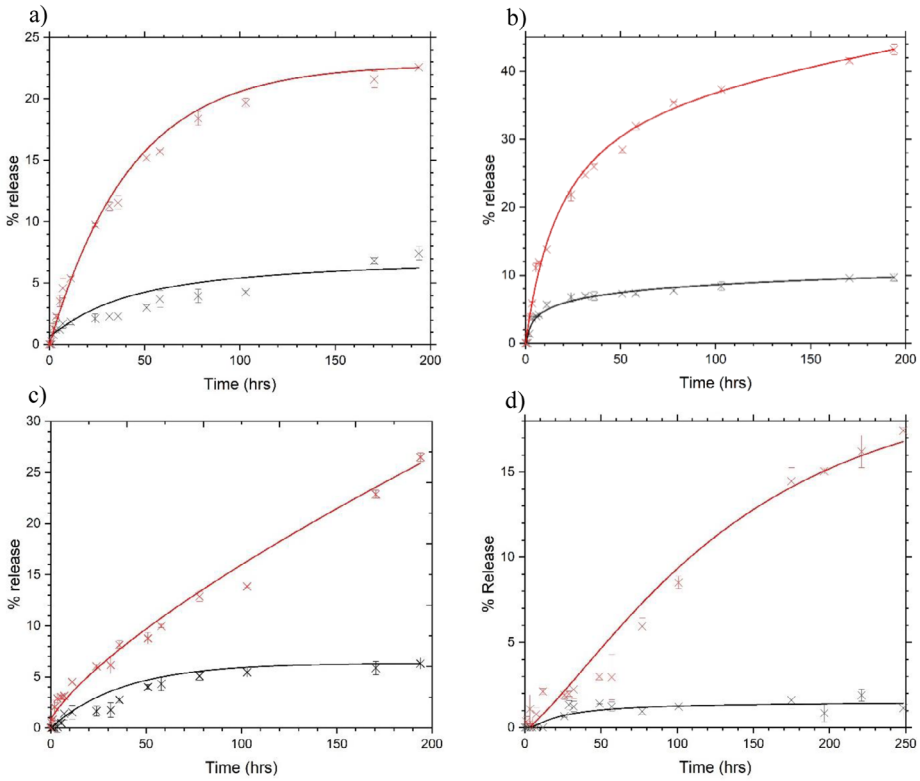


Figure 6. Release of Dox from nanoparticles created from a) PEG_{112}-*b*-$PGly_{14}$, b) PEG_{112}-*b*-$PGly_{20}$, c) PEG_{112}-*b*-$PAla_6$ and d) polymer 5, PEG_{112}-*b*-$PAla_{14}$, in response to incubation in acetate buffer at pH 5.0 (red) and PBS buffer at pH 7.4 (black).

192 h. The final amount of Dox released was 3.5, 4.6, 4.2 and 12.4 times greater in pH 5.0 solution compared to pH 7.4 solution for nanoparticles of polymers 4, 5, 6, and 7, respectively, demonstrating the sensitivity of the nanoparticles to acidic conditions. These results provide the first evidence that the ROP of 2,5-DKPs may be exploited to form stimuli-responsive polymers capable of forming nanoparticles for controlled delivery applications. Monomer (2,5-DKP) synthesis is straightforward, the monomers are stable against hydrolysis and premature polymerization ensuring ease of storage, and polymerization from a mPEG macroinitiator is achievable, ensuring stable nanoparticle formation.

4. Conclusions

The synthesis of poly(amino acids) and poly(amino acid)-containing diblock copolymers via the ROP of amino acid 2,5-DKPs is reported for the first time. Although monomer conversion did not reach completion, rendering the polymerization non-living, a range of poly(amino acid) products were created. Poly(amino acids) have enormous potential for use as biomaterials owing to their inherent chemical functionality, environmental credentials, biocompatibility, and biodegradability. Polymer synthesis was achieved using DI water as the solvent, and the circularity of the process was demonstrated owing to PAla susceptibility to elastase hydrolysis, and the subsequent ease of 2,5-DKP synthesis from recovered Ala. 2,5-DKP ROP may be initiated by primary amine or alcohol groups, with the latter allowing the formation of amphiphilic block copolymers initiated from mPEG that readily formed stable nanoparticles. Such nanoparticles proved effective in encapsulating Dox, crucially maintaining the drug when stored in nonacidic solution. Conversely, prolonged Dox release occurred when the nanoparticles were stored in acidic solution (pH 5.0). It is anticipated that the creation poly(amino acids) from 2,5-DKPs will promote their widespread use in applications ranging from everyday materials to advanced healthcare devices.

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

P.A.W.: investigation, formal analysis, data curation, methodology, writing (original draft preparation, review & editing). C.O.H.S.: investigation, data curation, methodology, formal analysis. K.M.: investigation, data curation, methodology, formal analysis. P.D.T.: conception, supervision, project administration, data curation, and writing, review & editing.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords

2,5-diketopiperazine ring-opening polymerization, 2,5-diketopiperazines, biorenewable polymers, drug delivery, poly(amino acids), polymer recycling

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[1] A. D. Borthwick, *Chem. Rev.* **2012**, *112*, 3641.
[2] F. Jubeen, S. Ijaz, I. Jabeen, U. Aftab, W. Mehdi, A. Altaf, S. A. Alissa, H. A. Al-Ghulikah, S. Ezzine, I. Bejaoui, *Arabian J. Chem.* **2022**, *15*, 104299.
[3] C. Labrière, N. Kondori, J. S. Caous, M. Boomgaren, K. Sandholm, K. N. Ekdahl, J. H. Hansen, J. Svenson, *J. Pept. Sci.* **2018**, *24*, e3090.
[4] A. Mollica, R. Costante, S. Fiorito, S. Genovese, A. Stefanucci, V. Mathieu, R. Kiss, F. Epifano, *Fitoterapia* **2014**, *98*, 91.
[5] S. Manchineella, C. Voshavar, T. Govindaraju, *Eur. J. Org. Chem.* **2017**, *2017*, 4363.
[6] T. M. Grant, D. Rennison, H. Arabshahi, M. A. Brimble, P. Cahill, J. Svenson, *Org. Biomol. Chem.* **2022**, *20*, 9431.
[7] S. Manchineella, N. A. Murugan, T. Govindaraju, *Biomacromolecules* **2017**, *18*, 3581.
[8] Y. Ji, G.-C. Kuang, X.-R. Jia, E.-Q. Chen, B.-B. Wang, W.-S. Li, Y. Wei, J. Lei, *Chem. Commun.* **2007**, 4233.
[9] M. Khuphe, B. Mukonoweshuro, A. Kazlauciunas, P. D. Thornton, *Soft Matter* **2015**, *11*, 9160.
[10] R. D. Murphy, E. Bobbi, F. C. S. de Oliveira, S.-A. Cryan, A. Heise, *J. Polym. Sci., Part A: Polym. Chem.* **2019**, *57*, 1209.
[11] H. An, L. M. Zhu, J. F. Shen, W. J. Li, Y. Wang, J. L. Qin, *Colloids Surf., B* **2020**, *185*, 110601.
[12] M. Khuphe, C. S. Mahon, P. D. Thornton, *Biomater. Sci.* **2016**, *4*, 1792.
[13] B. S. McAvan, M. Khuphe, P. D. Thornton, *Eur. Polym. J.* **2017**, *87*, 468.
[14] R. Yang, X. Wang, S. Liu, W. Zhang, P. Wang, X. Liu, Y. Ren, X. Tan, B. Chi, *Int. J. Biol. Macromol.* **2020**, *142*, 332.
[15] H. Yu, N. Ingram, J. V. Rowley, D. C. Green, P. D. Thornton, *Chem. - Eur. J.* **2020**, *26*, 13352.
[16] J. V. Rowley, P. A. Wall, H. Yu, M. J. Howard, D. L. Baker, A. Kulak, D. C. Green, P. D. Thornton, *Polym. Chem.* **2022**, *13*, 100.
[17] P. Li, H. Zong, G. Li, Z. Shi, X. Yu, K. Zhang, P. Xia, S. Yan, J. Yin, *ACS Biomater. Sci. Eng.* **2023**, *9*, 4855.
[18] C. Zhang, J. Yuan, J. Lu, Y. Hou, W. Xiong, H. Lu, *Biomaterials* **2018**, *178*, 728.
[19] J. M. Palomo, *RSC Adv.* **2014**, *4*, 32658.
[20] A. Rasines Mazo, S. Allison-Logan, F. Karimi, N. J.-A. Chan, W. Qiu, W. Duan, N. M. O'Brien-Simpson, G. G. Qiao, *Chem. Soc. Rev.* **2020**, *49*, 4737.
[21] Y. Otake, H. Nakamura, S. Fuse, *Angew. Chem., Int. Ed.* **2018**, *57*, 11389.
[22] K. Koga, A. Sudo, T. Endo, *J. Polym. Sci., Part A: Polym. Chem.* **2010**, *48*, 4351.
[23] W. H. Daly, D. Poché, *Tetrahedron Lett.* **1988**, *29*, 5859.
[24] T. Endo, A. Sudo, *Polym. Int.* **2020**, *69*, 219.
[25] C. Capello, U. Fischer, K. Hungerbühler, *Green Chem.* **2007**, *9*, 927.
[26] H. J. Oh, M. K. Joo, Y. S. Sohn, B. Jeong, *Macromolecules* **2008**, *41*, 8204.
[27] S. Zhao, X. Ye, M. Wu, J. Ruan, X. Wang, X. Tang, B. Zhong, *Int. J. Mol. Sci.* **2021**, *22*, 1513.
[28] P. von Tiedemann, S. Anwar, U. Kemmer-Jonas, K. Asadi, H. Frey, *Macromol. Chem. Phys.* **2020**, *221*, 1900468.
[29] U.S. Food and Drug Administration, CFR – Code of Federal Regulations Title 21, **2020**, https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=175.300 (accessed: December 2023).
[30] M. Ryner, K. Stridsberg, A.-C. Albertsson, H. Von Schenck, M. Svensson, *Macromolecules* **2001**, *34*, 3877.
[31] M. Khuphe, A. Kazlauciunas, M. Huscroft, P. D. Thornton, *Chem. Commun.* **2015**, *51*, 1520.
[32] S. E. Blondelle, B. Forood, R. A. Houghten, E. Pérez-Payá, *Biochemistry* **1997**, *36*, 8393.
[33] O. S. Rabotyagova, P. Cebe, D. L. Kaplan, *Macromol. Biosci.* **2010**, *10*, 49.
[34] O. Rathore, D. Y. Sogah, *J. Am. Chem. Soc.* **2001**, *123*, 5231.
[35] G. J. M. Habraken, M. Peeters, P. D. Thornton, C. E. Koning, A. Heise, *Biomacromolecules* **2011**, *12*, 3761.
[36] M. Shahriari, M. Zahiri, K. Abnous, S. M. Taghdisi, M. Ramezani, M. Alibolandi, *J. Controlled Release* **2019**, *308*, 172.
[37] J. K. Patra, G. Das, L. F. Fraceto, E. V. R. Campos, M.d.P. Rodriguez-Torres, L. S. Acosta-Torres, L. A. Diaz-Torres, R. Grillo, M. K. Swamy, S. Sharma, S. Habtemariam, H.-S. Shin, *J. Nanobiotechnol.* **2018**, *16*, 71.
[38] L. Kostka, L. Kotrchova, V. Subr, A. Libanska, C. A. Ferreira, I. Malatova, H. J. Lee, T. E. Barnhart, J. W. Engle, W. Cai, M. Sirova, T. Etrych, *Biomaterials* **2020**, *235*, 119728.
[39] M. Khuphe, N. Ingram, P. D. Thornton, *Nanoscale* **2018**, *10*, 14201.
[40] R. de la Rica, D. Aili, M. M. Stevens, *Adv. Drug Delivery Rev.* **2012**, *64*, 967.
[41] M. Danaei, M. Dehghankhold, S. Ataei, F. H. Davarani, R. Javanmard, A. Dokhani, S. Khorasani, M. R. Mozafari, *Pharmaceutics* **2018**, *10*, 57.
[42] S. Senapati, A. K. Mahanta, S. Kumar, P. Maiti, *Signal Transduction Targeted Ther.* **2018**, *3*, 7.
[43] Y. Liu, W. W. Y., J. Yang, C. Zhou, J. Sun, *Asian J Pharm. Sci.* **2013**, *8*, 159.

