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Full Paper

Block Copolypeptide Nanoparticles for the Delivery of Ocular Therapeutics

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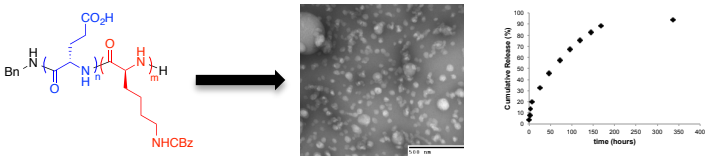
Self-assembling block copolypeptides were prepared by sequential ring-opening polymerization of N-carboxyanhydride (NCA) derivatives of γ-benzyl-L-glutamic acid and ε-carbobenzyloxy-L-lysine, followed by selective deprotection of the benzyl glutamate block. The synthesized polymers had number average molecular weights close to theoretical values, and had low dispersities ($Đ_M$ = 1.15-1.28). Self-assembly of the amphiphilic block copolymers into nanoparticles was achieved using the 'solvent-switch' method, whereby the polymer was dissolved in THF and water and the organic solvent removed by rotary evaporation. The type of nanostructures formed varied from spherical micelles to a mixture of spherical and worm-like micelles, depending on copolymer composition. The spherical micelles had an average diameter of 43 nm by dynamic light scattering, while the apparent diameter of the mixed phase system around 200 nm. Reproducibility of nanoparticle preparation was demonstrated to be excellent; almost identical DLS traces were obtained over three repeats. Following qualitative dye-solubilisation experiments, the nanoparticles were loaded with the ocular anti-inflammatory drug dexamethasone. Loading efficiency of the

nanoparticles was 90% and the cumulative drug release was 94% over 16 days, with a 20% burst release in the first 24 hours.

FIGURE FOR ToC_ABSTRACT



1. Introduction

Disorders affecting the posterior segment of the eye, which cause visual impairment and blindness, are occurring at an increasing rate[1]. For example, it has been estimated that within a few years ca. 30 million Americans will be affected by age-related macular degeneration (AMD). Injection of drugs into the vitreous cavity (intravitreal injection) delivers therapeutics to the target site, however, when the disease is chronic, such as with uveitis, macular edema and AMD, frequent injections are required to achieve intraocular drug levels within the therapeutic range. This can cause severe secondary effects such as cataracts, retinal detachment, endophthalmitis and intravitreal haemorrhages. Even though intravitreal injection is accepted clinically, patient compliance is poor and the risk of complications increases dramatically as the number of required injections increases with the age of the patient. An effective drug delivery platform is required to solve this problem.

Currently, micro- and nanoparticles prepared from linear, biodegradable polyesters such as poly(lactic-co-glycolic acid) (PLGA) are used as intraocular drug delivery systems.[2] However, polymer degradation produces acidic species that can cause inflammation. In addition, chemical functionalisation of the particle surface is non-trivial and drug release cannot be triggered. Self-assembling peptide-based materials have a number of attractive features in the context of nanoparticles for drug delivery[3]: their biodegradation produces non-inflammatory amino acids; rich chemical diversity allows easy attachment of, inter alia, targeting vectors and species to improve biocompatibility; self-assembly in aqueous media is compatible with the loading of sensitive cargoes such as growth factors, proteins, biopharmaceuticals and nucleotides.

An efficient method for preparing self-assembling polypeptides is by the polymerisation of N-carboxyanhydride (NCA) derivatives of a-amino acids[3-5]. Controlled polymerisation of

NCAs to produce well-defined polypeptides of desired molecular weight and narrow dispersity can be achieved using a variety of species including primary amines[6], certain transition metal complexes[7, 8] and N-trimethylsilylamines[9, 10]. When using primary amines as initiators, a number of strategies to improve polymerisation control, including lower reaction temperatures[11, 12], the use of ammonium salts as dormant initiators[13, 14] and the removal of CO_2 by purging the reaction vessel with inert gas[15], have been employed. In our work, we use TMS-amines developed by Cheng et al.[9, 10] as initiators, since polymerisations are generally more rapid and give better control than when primary amines are used. Here we report the preparation of nanoparticles from block copolymers of poly(L-glutamic acid) and poly(ε-carbobenzyloxy-L-lysine), synthesised by sequential NCA polymerization of benzyl-L-glutamate (BnE) and Z-lysine (ZK) NCAs, followed by deprotection of the benzyl-L-glutamate block (**Figure 1**). We chose to deprotect the BnE rather than the ZK block since poly(L-lysine) is known to be cytotoxic. Poly(glutamic acid-b-lysine) block copolymers have been prepared previously from NCA polymerization by Lecommandoux et al. and were shown to assemble into a variety of nanostructures[16, 17], but only at low (<4) or high (>10) pH values which are not suitable for drug delivery applications.

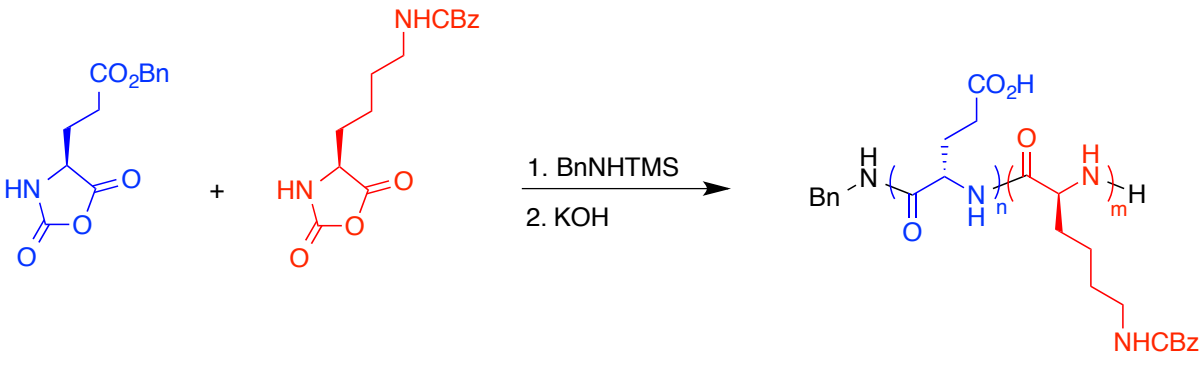


Figure 1. Synthesis of amphiphilic poly(L-glutamic acid)-*b*-(ε-carbobenzyloxy-L-lysine).

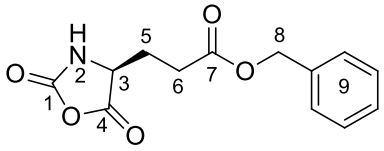
2. Experimental Section

2.1. Materials and Instrumentation

H-Glu(Bn)-OH and H-Lys(Z)-OH were purchased from Novabiochem. Alpha-pinene was purchased from Sigma Aldrich. Triphosgene was purchased from Alfa Aesar. N-Benzyltrimethylsilylamine was purchased from TCI. Dexamethasone ≥ 98% was obtained from Sigma-Aldrich. Acetonitrile (ACN; HPLC grade), dichloromethane (DCM; laboratory grade) and phosphate buffered saline (PBS) were obtained from Fisher Scientific and distilled H_2O from a high purity source. PBS was obtained in the form of tablets: one tablet is to dissolve in 100 ml dist. H_2O (one tablet consists of 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl and pH 7.3-7.5). DMF (anhydrous) was purchased from Sigma Aldrich. THF was purchased from Fischer Scientific and dried by passage through two alumina columns using an Innovative Technology Inc. solvent purification system and stored under nitrogen. THF was further dried over sodium/ benzophenone complex with at least 3 freeze thaw cycles until the purple colouration persisted. Float-A-Lyzer® dialysis cassettes (MWCO 20 kDa; size: 1 ml) were obtained from Sigma-Aldrich Cronus and nylon 0.45 μm filters and Millex MP 0.22 μm filters were obtained from Fisher Scientific. NMR spectra were recorded using a Varian Inova 500 spectrometer at 499.87 (^{1}H), a Varian VNMR-700 spectrometer at 699.73 MHz and a Bruker Advance 400 spectrometer at 400.13 MHz (^{1}H) or 100.26 (^{13}C). NMR spectra were analyzed using MestRE Nova 6.2 software. Gel permeation chromatography was undertaken with THF and DMF as solvents. 100 μL of solution agitated overnight to ensure complete dissolution was injected at a flow rate of 1.00 ml min^{-1} by a Viscotek SEC autochanger model. A Viscotek TDA 301 unit with triple detection (right angle laser scattering at 670 nm, differential refractometer and viscometer) was used. Analyses were undertaken using OmniSEC 4.0 software. Dynamic light scattering was conducted on a Brookhaven ZetaPlus combined DLS and zeta potential analyzer. Particle size was determined using BIC Particle

sizing software. TEM was carried out using an Hitachi H-7600 operating at 100 kV, the TEM camera system was operated using AMT 600 software. One drop (10 ml) of prepared aggregate solution was placed on a glow discharged (20 seconds), carbon coated, 400 mesh copper grid for 30 seconds, blotted with filter paper and stained with 1% aqueous uranyl acetate for 30 seconds. Nanoparticles were prepared using a Büchi Rotavapor R-215 consisting of a Vacuum controller V-855 and Heating bath B-491. Dexamethasone loading and release were determined by HPLC using a Perkin Elmer HPLC Series 200 with a pump (Perkin Elmer series 200), a UV/VIS detector (Perkin Elmer series 200), a vacuum degasser (Perkin Elmer series 200), an auto sampler (Perkin Elmer series 200) and Total Chrom 6.3.1 HPLC Software. The chromatographic separation was achieved with a C18 column Tracer Excel 120 ODSA (particle size 5 μm, 150 mm x 4mm; Teknokroma S. Coop., Barcelona, Spain).

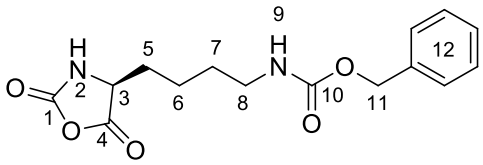
2.2. Synthesis of γ-Benzyl-L-glutamic acid (BnE) NCA



To a stirred suspension of H-Glu(Bn)-OH (2.00 g, 8.43 mmol) in 20 mL of anhydrous THF under an atmosphere of nitrogen at 50 °C was added triphosgene (0.84 g, 2.81 mmol) in one portion and stirred to dissolution. The reaction was stirred under a flow of nitrogen for 3 h at 50 °C. When solid starting material remained after one hour, an aliquot (0.05 equiv.) of triphosgene was added and this was repeated every half an hour if there was still solid left. After this time the reaction mixture was allowed to cool down to room temperature. The reaction mixture was added to ice-cold (-18 °C) hexane (60 mL) and the precipitate recrystallized twice from THF/hexane at -18 °C. The white solid was obtained by centrifugation following each crystallization, and finally dried under high vacuum overnight to afford BnE NCA (1.62 g; 73% yield) as a white powder. ^{1}H NMR (500 MHz, $CDCl_3$, δ)

7.36 (m, 5H, H^9), 6.26 (s, 1H, H^2), 5.14 (s, 2H, H^8), 4.37 (m, 1H, H^3), 2.61 (t, J = 6.5 Hz, 2H; H^6), 2.29 (m, 1H, H^{5a}), 2.13 (m, 1H, H^{5b}). ^{13}C NMR (125 MHz, ^1H-decoupled, $CDCl_3$, δ): 172.3 (C^7), 169.1 (C^4), 151.2 (C^1), 134.9 (C^9), 128.6 (C^9), 128.5 (C^9), 128.2 (C^9), 67.0 (C^8), 56.9 (C^3), 29.9 (C^6), 26.8 (C^5). IR (film): ν = 3327, 1855 (C=O amide), 1774 (C=O anhydride), 1715 (C=O ester), 1241, 1194, 1104, 937, 735, 618 cm^{-1}.

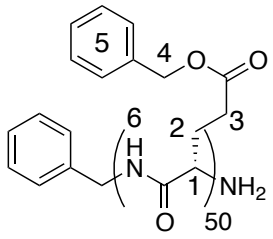
2.3. Synthesis of ε-Carbobenzyloxy-L-Lysine (ZK) NCA



To a stirred suspension of H-Lys(Z)-OH (2.00 g, 7.13 mmol) in 20 mL of anhydrous THF under an atmosphere of nitrogen at 50 °C was added triphosgene (0.71 g, 2.38 mmol) in one portion and stirred to dissolution. The reaction was stirred under a flow of nitrogen for 3 h at 50 °C. When solid starting material remained after one hour, an aliquot (0.05 equiv.) of triphosgene was added and this was repeated every half an hour if there was still solid left. After this time the reaction mixture was allowed to cool down to room temperature. The reaction mixture was added to ice-cold (-18 °C) hexane (60 mL) and the precipitate recrystallized twice from THF/hexane at -18 °C. The white solid was obtained by centrifugation following each crystallization, and finally dried under high vacuum overnight to afford ZK NCA (2.02 g; 93% yield) as a white powder. ^1H NMR (500 MHz, $CDCl_3$, δ): 7.36 (m, 5H, H^{12}), 6.64 (s, 1H, H^2), 5.11 (s, 2H, H^{11}), 4.89 (s, 1H, H^9), 4.28 (s, 1H, H^3), 3.20 (m, 2H, H^8), 1.99 (m, 1H, H^{5a}), 1.82 (m, 1H, H^{5b}), 1.56 (m, 4H, $H^{6,7}$). ^{13}C NMR (125 MHz, ^1H-decoupled, $CDCl_3$, δ): 168.8 (C^4), 156.0 (C^{10}), 151.2 (C^1), 135.3 (C^{12}), 127.6 (C^{12}), 127.3 (C^{12}), 127.1 (C^{12}), 66.0 (C^{11}), 56.4 (C^3), 38.9 (C^8), 29.6 (C^5), 28.1 (C^7), 20.0 (C^6). IR (film): ν

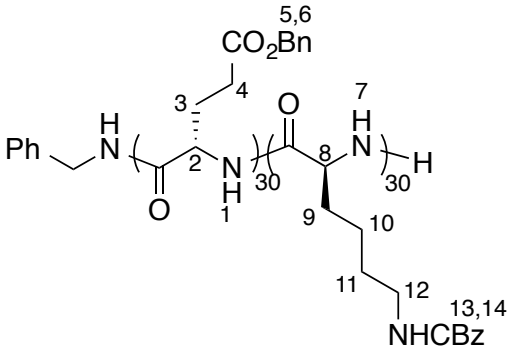
= 3335, 1852 (C=O amide), 1804 (C=O anhydride), 1766 (C=O ester), 1680, 1248, 1136, 941, 750, 688 cm^{-1}.

2.4. Synthesis of Poly(γ-benzyl-L-glutamic acid) (PBnE)



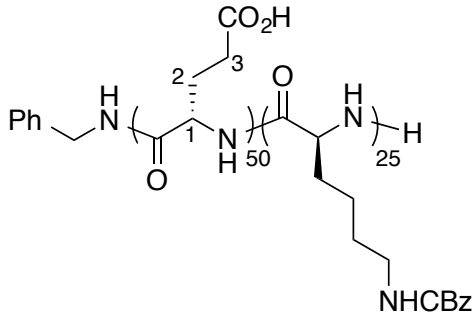
γ-Benzyl-protected L-glutamic acid NCA (0.5 g, 1.89 mmol) was dissolved in anhydrous DMF (10 ml). A Schlenk tube was preflushed with nitrogen gas before the dissolved NCA compound was added. N-Benzylamine TMS (0.038 mmol) was added dropwise to the Schlenk tube. The reaction was stirred for 24 hours at room temperature. To stop the reaction, the solution was added dropwise to diethyl ether (190 ml). The product was stored overnight at -8 ˚C for precipitation. After complete precipitation the sample was centrifuged to obtain a white solid. Further purification was carried out by re-dissolving in THF following by precipitation in 20-fold excess of diethyl ether. The purification was repeated one further time. The final pure product was dried under high vaccum overnight to obtain a white powder (0.551 g, 66% yield). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.33 (1H, H^6), 7.26 (5H, H^5), 5.04 (2H, H^4), 3.94 (1H, H^1), 2.58 (2H, H^3), 2.29 (1H, $H^2{}_b$), 2.13 (1H, $H^2{}_a$). IR: ν = 3298, 3034 (aromatic ring), 2960, 1730 (C=O carboxylic ester), 1650 (C=O ketone), 1544, 1452, 1164 (C-O carboxylic ester), 976, 742, 696, 610, 508 cm^{-1}. GPC (THF): M_n 11,100; $Đ_M$ 1.15.

2.5. Synthesis of γ-Benzyl-L-glutamate / ε-Carbobenzyloxy-L-lysine Block Copolymers (PBnE-b-ZK)



Block copolypeptides were prepared by sequential monomer addition without intermediate purification steps. Different target molecular weights and block ratios were obtained by varying the monomer to (macro)initiator ratio. The following aims to produce the block copolymer $PBnE_{30}$-b-ZK_{30} and serves as a general procedure: BnE NCA (100 mg, 0.38 mmol) was dissolved in 1.5 mL of dry DMF, and injected into a Schlenk tube fitted with a Young's tap and 9mm rubber septum pre-filled with nitrogen. N-Benzyltrimethylsilylamine in DMF (127 µL, 0.1M, 0.0127 mmol) was added via syringe into the tube and allowed to stir for 24 h. Following this a 30 µL sample was withdrawn and diluted to give a 1 mg/mL solution for GPC analysis. ZK NCA (137 mg, 0.38 mmol) was dissolved in 1.5 mL of dry DMF, and via syringe added to the reaction mixture. After 24 h of additional stirring a 30 µL sample was withdrawn and diluted to give a 1 mg/mL solution for GPC analysis and the remainder of the reaction was poured into a 20-fold excess of diethyl ether with stirring. The precipitated solid was collected by centrifugation. Further purification was achieved by re-dissolving in THF and precipitating into a 20-fold excess of diethyl ether to afford the block copolypeptide (yield 184 mg, 91%) as a white powder. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 8.10-8.50 (m, 2H, $H^{1,7}$), 7.10-7.40 (m, 10H, $H^{6,15}$), 5.50 (m, 1H, H^{13}), 4.90-5.20 (m, 4H, $H^{5,14}$), 3.80-4.00 (m, 2H, $H^{2,8}$), 3.00-3.20 (m, 2H, H^{12}), 1.80-2.70 (m, 4H, $H^{3,4}$), 1.30-1.70 (m, 6H, $H^{9,10,11}$). GPC (DMF): M_n 15,800; $Đ_M$ 1.17.

2.6. Deprotection of Poly(γ-benzyl-L-glutamate)-*b*-(ε-carbobenzyloxy-L-lysine)



The block copolymers were dissolved in THF (0.025 g ml^{-1}). Potassium hydroxide (1.5 mol equivalent per benzyl ester function) was dissolved in a minimum of H_2O and added to the reaction mixture. The reaction was stirred for 15 hours before it was added dropwise to an excess of diethyl ether. The product precipitated and it was stored for 1 hour at -8 ˚C. The mixture was centrifuged and the supernatant was removed. A further washing step was carried out before the product was obtained as a white powder (46% yield for PE_{50}-*b*-ZK_{25}, 66% yield for PE_{50}-*b*-ZK_{50}).

^{1}H NMR of PE_{50}-*b*-ZK_{25} (400 MHz, D_2O, δ): 4.34 (1H, H^1), 2.26 (2H, H^3), 1.84 – 2.15 (2H, H^2).

^{1}H NMR of PE_{50}-*b*-ZK_{50} (400 MHz, D_2O, δ): 4.30 (1H, H^1), 2.23 (2H, H^3), 1.81 – 2.14 (2H, H^2).

2.7. Self-assembly

The polymers were first dissolved in THF at a concentration of 1.0 mg ml^{-1}. Filtered MilliQ water was added to the solution and it was stirred overnight, allowing the THF to evaporate. If necessary, the solutions were finally filtered through a 0.45 μm filter. The final polymer concentration in water was 1.0 mg ml^{-1}.

2.8. Nile Red encapsulation

Polymers were dissolved in a solution of 0.02 mg ml^{-1} of Nile Red in THF resulting in a dissolved polymer concentration of 1.0 mg ml^{-1}. After successful dissolution, filtered MilliQ water was added and it was stirred overnight to allow the THF to evaporate. The final polymer concentration in water was 1.0 mg ml^{-1}.

2.9. Dexamethasone Encapsulation and Release

The nanoparticles loaded with dexamethasone (DX) were prepared following this procedure: 10 mg of the polymer were dissolved in 5 ml solution of 0.1 mg ml^{-1} DX in THF and 5 ml dist. H_2O was added. First the solution was vortexed for 30-60 s and then was sonicated for a further 5-15 min depending on the solubility. The solution was condensed under reduced pressure using a Büchi rotary evaporator with the following settings: 15 min at 200 mbar pressure (Δ pressure 5 mbar), rotation 150 rpm and water bath at 23 °C, then a further 15 min at 100 mbar pressure (Δ pressure 5 mbar), rotation 150 rpm and water bath at 23 °C. The nanoparticles were filtered with a 0.22 µm Millex MP, pipetted into an Eppendorf tube and then lyophilised overnight. 1 mg of the lyophilised nanoparticles were resuspended in 1 ml of a filtered PBS solution (with a Cronus and nylon 0.45 µm) and further characterized (TEM and DLS) and used for the DX release. The DX loaded nanoparticles (1 ml) were pipetted into a pre-treated dialysis cassette (washed first with 10% isopropanol then dist. H_2O and finally with filtered PBS) and enclosed by 30 ml of filtered PBS in a falcon tube and incubated under gentle agitation at 37 °C. At different time points (1 h, 3 h, 5 h, 7 h, 24 h, 48 h, 4 d, 6d, 8 d, 10 d, 12 d, 14d and 16), a 10 ml sample of the PBS was taken and replaced with freshly filtered PBS and further analysed by HPLC with UV detection at 254 nm. For analysis of the DX encapsulation efficiency 5 mg of nanoparticles were dissolved in 200 µl DCM and then 800 µl of ACN were added and after strong vortexing for 2 minutes the solution was centrifuged

at 13,000 rpm for 20 min. Thereafter the supernatant was removed, filtered through a Millex MP 0.22 µm filter and analysed analysed by HPLC with UV detection at 254 nm.

The composition of the mobile phase A was acetonitrile:water (35:65 v/v). The mobile phase B was composed by 100% acetonitrile. Gradient elution method:

Step	min (duration)	A %	B%	slope
0	1	100	0	
1	10	100	0	
2	5	0	100	1
3	5	100	0	1
4	5	100	0	
5	1	100	0	

The mobile phase flow was set at 1 ml min^{-1} and the injection volume was 150 µl. After equilibration with the solvent to obtain a stable baseline, aliquots of samples were injected. The absorbance of the eluent was monitored at 254 nm with a detection sensitivity of 0.10 aufs. All analyses were performed at 25ºC.

For analysis of DX encapsulation efficiency, stock solutions of DX were prepared by dissolving 5 mg of DX in 25 ml in DCM:ACN (2:8) (0.2 mg ml^{-1}). Solutions were protected from exposure to light. The resulting solutions were then diluted with DCM:ACN (2:8) to give drug concentrations of 2 µg ml^{-1}, 5 µg ml^{-1}, 10 µg ml^{-1}, 15 µg ml^{-1} and 20 µg ml^{-1}. For analysis of DX release, stock solutions of DX were prepared by dissolving 5 mg of DX in 25 ml of PBS (0.2 mg ml^{-1}). Solutions were protected from exposure to light. The resulting solutions were then diluted with PBS to give drug concentrations of 2 µg ml^{-1}, 5 µg ml^{-1}, 10

μg ml^{-1}, 15 μg ml^{-1} and 20 μg ml^{-1}. The assay was validated with respect to linearity, accuracy and reliability in the range of concentrations of 2-20 μg ml^{-1}.

Encapsulation efficiency was calculated *via* following equation:

$$\text{Drug encapsulation efficiency (\%, w/w)} = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100$$

The drug loading of drug was calculated by the following equation:

$$\text{Drug loading (\%, w/w)} = \frac{\text{Actual drug loading}}{\text{overall mass of NPs}} \times 100$$

3. Results and Discussion

For the synthesis of amphiphilic polymers, a hydrophilic and a hydrophobic monomer is required. We decided to use γ-benzyl-protected L-glutamic acid (BnE) and ε-(benzyloxycarbonyl)-L-lysine (ZK) as the parent amino acids. Although these monomers will initially form hydrophobic diblock copolymers, either amino acid can be deprotected to produce an amphiphilic block copolymer product. Triphosgene is the most widely used compound to create *N*-carboxyanhydride (NCA) derivatives for ring-opening polymerisation (ROP). In this work, the procedure according to Daly and co-workers was followed[18]. After two hours reaction time in THF, full conversion of the amino acids into NCAs was observed. All reaction mixtures became clear solutions and the products were recrystallized several times to remove the by-product, HCl. As a consequence, yields of these NCAs were high using the exact conditions as described by Daly and co-workers.

Homopolymerization of BnE NCA was carried out using TMS-protected benzylamine as initiator, giving a high degree of control over the reaction. The predicted number average

molecular weight (M_n) agrees very well with the observed value and the low dispersity value ($Đ_M$ = 1.15) indicates a controlled polymerization (**Table 1**). Therefore, block copolymerizations were also carried out using this initiator. It was found that isolation and purification of the first BnE block before attempting chain extension with ZK NCA did not lead to well-defined block copolymers (data not shown). Consequently, the ZK NCA was added directly to the reaction medium after complete consumption of the BnE NCA. In this manner, block copolypeptides with either a 1:1 or 2:1 target block ratio between BnE and ZK were synthesized successfully. Before the addition of ZK NCA a small sample was removed for GPC analysis, to investigate the control of the polymerization of the first block. Determination of the poly-Z-protected lysine (PZK) block length was carried out by NMR-peak integration measurements. All block copolymers showed good to excellent control over the polymerization. The $Đ_M$ values ranged from 1.12 to 1.28 for polymers with a ratio of 2:1 and the polymer with a ratio of 1:1 showed a low PDI of 1.27.

Table 1. Characterisation Data for Poly(γ-benzyl-L-glutamic acid)-*b*-(ε-carbobenzyloxy-L-lysine) Copolymers

Sample	**Expected M_n [Da]**	**Obtained M_n [Da]**	**$Đ_M$**	**Block ratio (E:K)[a)]**	**Yield [%]**
$PBnE_{50}$	11,057	11,100	1.15	n/a	66
$PBnE_{30}$-*b*-ZK_{30}	14,700	15,800[b)]	1.17	49:51	91
$PBnE_{30}$-*b*-ZK_{15}	10,607	14,500[b)]	1.17	60:40	88
$PBnE_{50}$-*b*-ZK_{50}	24,157	30,500[c)]	1.27	50:50	68
$PBnE_{50}$-*b*-ZK_{25}	17,607	20,400[c)]	1.28	71:29	72

[a)] determined by ^{1}H NMR spectroscopy; [b)] determined by GPC using DMF as eluent; [c)] M_n of first block determined by GPC (THF eluent); total M_n estimated by GPC and ^{1}H NMR spectroscopy.

Block copolypeptide products were converted into amphiphilic polypeptides by hydrolysis of the benzyl protecting group on the glutamate block. This strategy was favoured over the alternative lysine side-chain deprotection route since polylysine is known to be cytotoxic[19]. Cleavage of the benzyl protecting group was achieved under basic conditions using 1.5 equivalents of potassium hydroxide in THF per benzyl ester function[20]. After two precipitation steps in diethyl ether and drying under high vacuum, the amphiphilic block copolypeptide product was analyzed by ^{1}H-NMR spectroscopy in D_2O. Since the amphiphilic product self-assembles in this medium, it was suggested that the deprotected poly(glutamic acid) (PGA) block would be seen in the spectrum. Indeed, a nearly complete absence of aromatic peaks was observed (**Figure 2**). This indicates that the aromatic rings of carbobenzyloxy protecting groups may be located in the core of micelles and therefore cannot be detected by the magnetic field. ^{1}H-NMR measurements in DMSO permit the analysis of both blocks and confirmed the presence of carbobenzylic protecting groups. Further analysis of integral ratios showed that the present aromatic peaks belong only to the carbobenzylic group resulting in the formation of amphiphilic PGA-b-PZK block-copolymers.

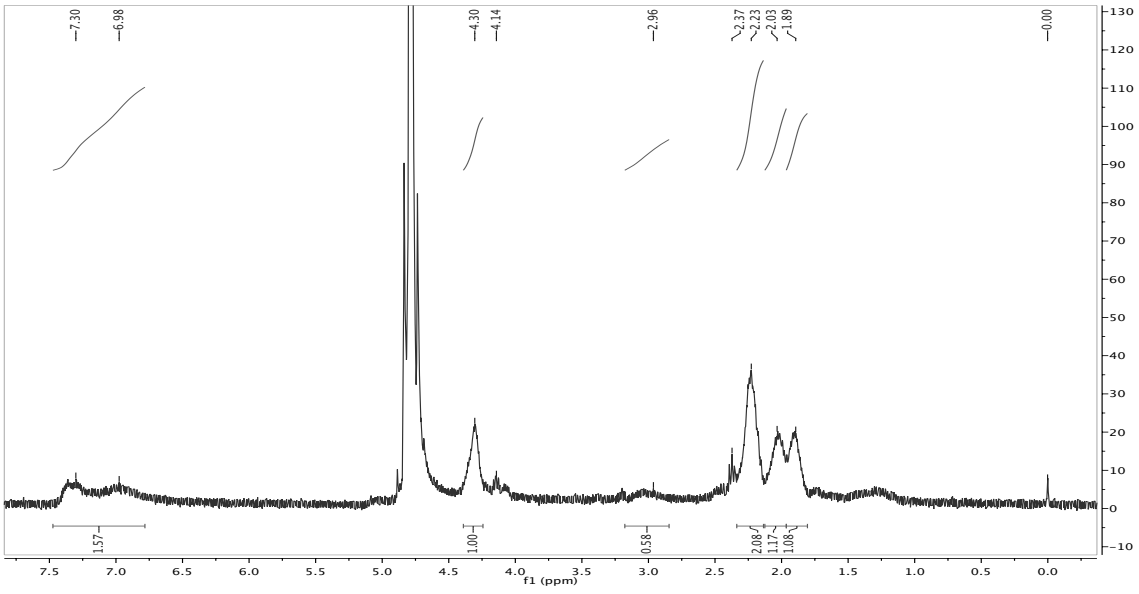


Figure 2. ^{1}H NMR spectrum of poly(L-glutamic acid)-*b*-(ε-carbobenzyloxy-L-lysine) in D_2O following deprotection of the glutamate block with KOH.

Table 2. Data for Poly(L-glutamic acid)-*b*-(ε-carbobenzyloxy-L-lysine) Copolymer Self-assembly

Sample	**Particle diameter [nm]**	**Zeta potential [mV]**	**CMC [g ml^{-1}]**
PE_{50}-*b*-ZK_{25}	207	-155	$1x10^{-3}$
PE_{50}-*b*-ZK_{50}	43	-155	$1x10^{-2}$

The solvent evaporation method was used to obtain self-assembled nanoparticles from PE-*b*-ZK block copolymers. The polymer was dissolved in THF and the same volume of water was added, followed by evaporation of the THF overnight. In all measurements, 1 mg ml^{-1} polymer concentration was used to create nanoparticles. Both block copolypeptides, PE_{50}-*b*-ZK_{25} and PE_{50}-*b*-ZK_{50} were self-assembled in this manner, and the diameter of the nanoparticles as determined by dynamic light scattering were found to be 207 nm and 43 nm, respectively (Table 2; **Figure 3D**). DLS measurements do not provide information about the morphology of the assembled particles, therefore TEM images were also obtained (Figure 3). Sample PE_{50}-*b*-ZK_{25} shows the presence of both spherical and worm-like particles (Figure 3A). The spherical particles have a diameter of around 30 nm, whereas the worm-like structures are much larger. PE_{50}-b-ZK_{50} also shows both spheres and worms, but appears more homogeneous with fewer worm-like structures (Figure 3B). This could explain the difference in DLS diameter between the two samples. PE_{50}-*b*-ZK_{25} has a larger apparent diameter by DLS due to the greater number of large, worm-like structures. Both sets of particles further showed a zeta potential of -155 mV that indicates the presence of the hydrophilic unprotected PGA block at the outer surface of the particles.

Due to their morphology and size, these nanoparticles may be suitable for drug delivery applications. Therefore, encapsulation of the hydrophobic dye Nile Red as a model 'drug' was investigated (Figure 3D). After Nile Red was dissolved in THF, the same amount of

polypeptide was added. Water was added after complete dissolution of the polypeptide and the THF was evaporated, causing the polypeptide to self-assemble and forcing the hydrophobic dye into the cores of the self-assembled nanostructure. The encapsulated dye turns the solution from hazy white into an almost clear light purple solution, indicating successful self-assembly and encapsulation. Interestingly, the size of particles is reduced significantly in the presence of the hydrophobic dye. Nile Red encapsulated samples showed a diameter reduction of 50% or more. It is suggested that the hydrophobic dye forces hydrophobic ends of the polypeptides into the core, thereby shrinking the size of the particles.

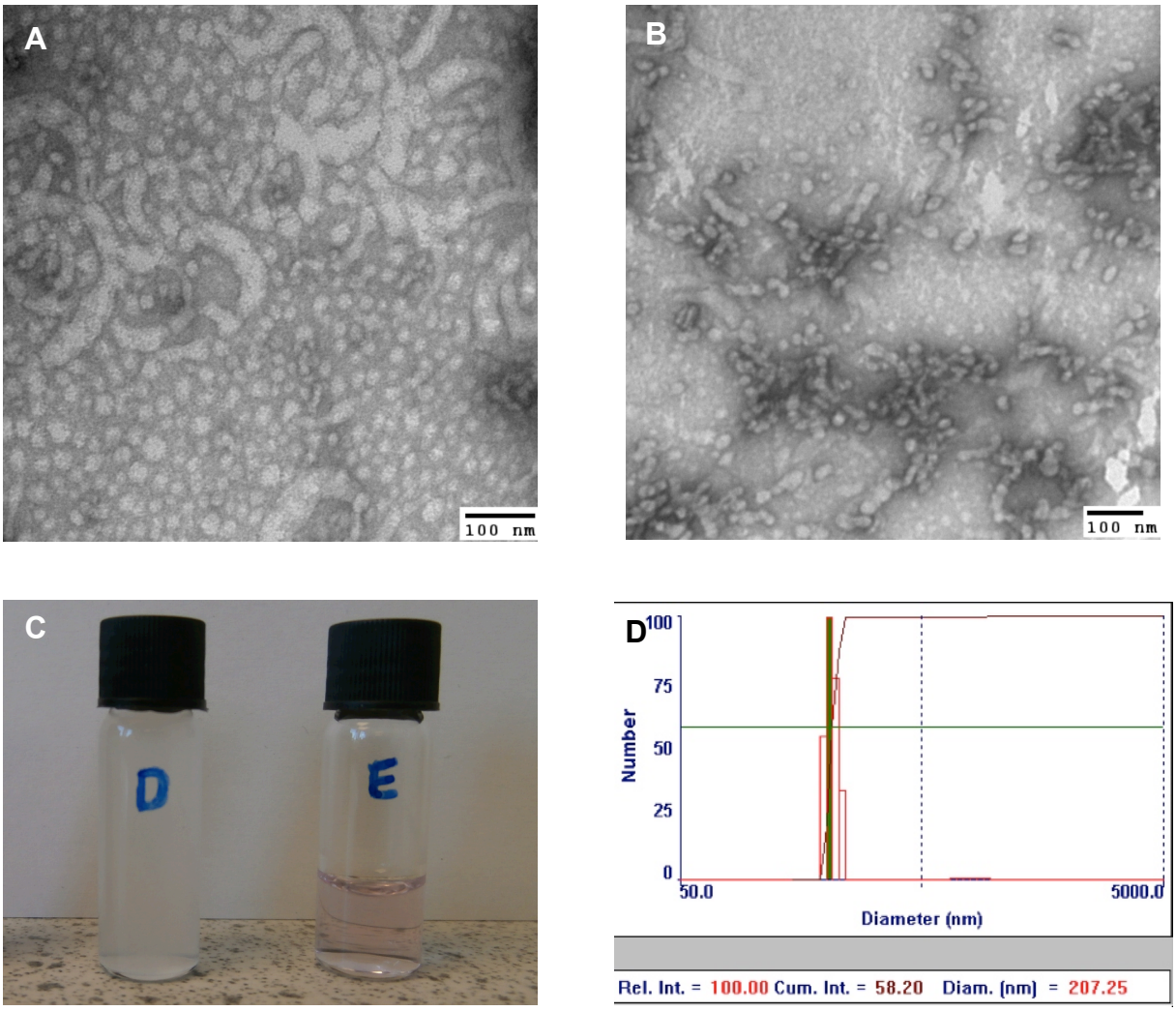


Figure 3. Self-assembly of PE-b-ZK copolymers: a) TEM of PE_{50}-*b*-ZK_{25}; b) TEM of PE_{50}-*b*-ZK_{50}; c) encapsulation of Nile Red by self-assembled nanostructures from PE_{50}-*b*-ZK_{50} (left –

without Nile Red; right – with Nile Red); d) DLS data for self-assembled nanostructures from PE_{50}-*b*-ZK_{25}.

Following successful encapsulation of Nile Red, we next explored the encapsulation and release of the anti-inflammatory ocular drug dexamethasone (DX). Nanoparticles from PE_{50}-*b*-ZK_{25} were prepared in the presence of DX (polymer:DX = 1:0.75) by the solvent evaporation approach using a controlled method involving defined periods of reduced pressure and temperature. The resulting nanoparticles were filtered to remove any large ill-defined aggregates, isolated by freeze-drying and resuspended in buffer. Repeated preparation of the DX-loaded nanoparticles showed excellent reproducibility, giving an average particle diameter of around 180 nm (**Figure 4**). The release of DX through a dialysis membrane was then monitored at 37 ^{o}C over 16 days (polymer:Dx = 20:1). Prior to release, a small portion of the loaded nanoparticles was precipitated into acetonitrile and the DX loading was determined by HPLC, giving a drug loading of 5.5 wt% and a loading efficiency of 90%. The release profile is shown in **Figure 5**. Around 20% burst release of dexamethasone is observed in the first 24 hours, after which the release is steady over the remaining period of the study. After 16 days, 94% of the loaded dexamethasone had been released.

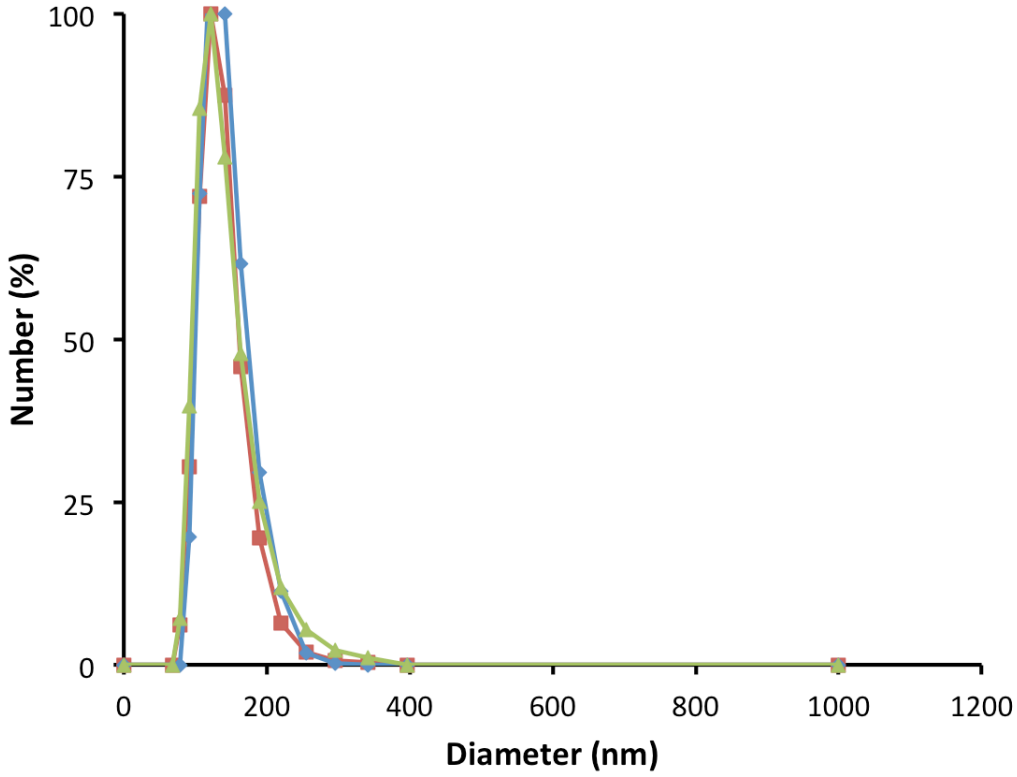


Figure 4. DLS traces of 3 repeat preparations of nanoparticles from PE_{50}-*b*-ZK_{25} (polymer concentration 1 mg/ml) loaded with dexamethasone (0.75 mg ml^{-1}).

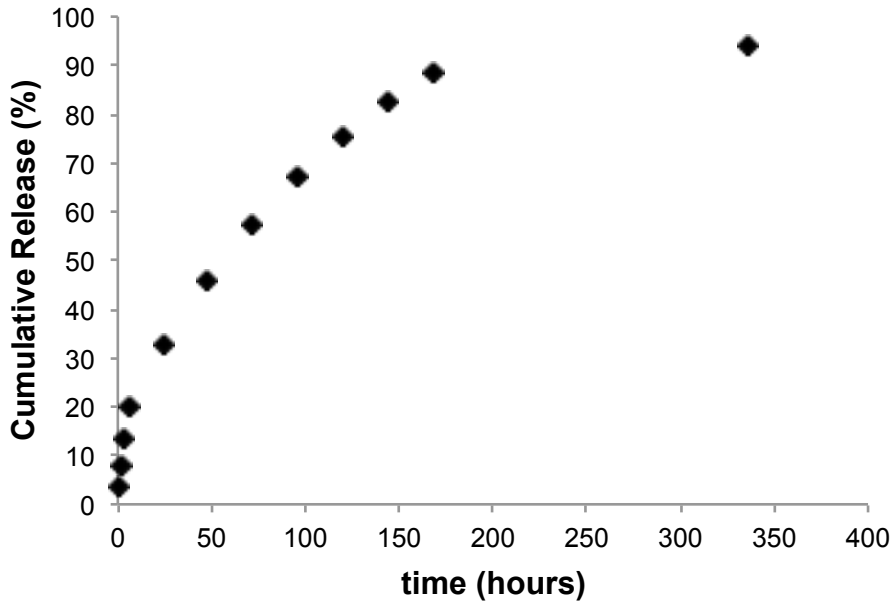


Figure 5. Release of dexamethasone from PE_{50}-*b*-ZK_{25} nanoparticles.

4. Conclusions

Well-defined amphiphilic block copolypeptides were prepared by sequential NCA ring-opening polymerization and were shown to self-assemble into nanoparticles in aqueous solution at pH 7. The nanoparticle morphology was dependent on composition: spherical micelles were found in the case of the PE_{50}-b-ZK_{50} polymer, whereas a mixture of spherical and worm-like micelles was found with the polymer PE_{50}-b-ZK_{25}. A protocol for nanoparticle preparation was developed, giving reproducible nanoparticles with a consistent diameter over three repeats. Successful encapsulation of the dye Nile Red demonstrated the ability of the nanoparticles to be loaded with hydrophobic molecules. Consequently, the loading and release of the hydrophobic ocular drug dexamethasone was investigated. The drug could be loaded with 90% efficiency at 5.5% of total nanoparticle mass, and 94% of the drug was released over 16 days in aqueous medium at 37 ^{o}C. These results demonstrate the potential for self-assembled block copolypeptides to act as nanoscale drug delivery vehicles for application in the eye.

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Nanoparticles from amphiphilic block copolypeptides are loaded with the ocular drug dexamethasone. The block copolypeptides are synthesized by sequential polymerization of benzyl-protected glutamic acid and Z-protected lysine *N*-carboxyanhydrides (NCAs). Selective deprotection of the benzyl glutamate block yields amphiphilic block copolymers that self-assemble into nanoaprticles in aqueous solution. Drug release is almost complete after 16 days in aqueous solution at 37 ^{o}C.

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Block Copolypeptide Nanoparticles for the Delivery of Ocular Therapeutics