Supplementary Information (SI) for RSC Pharmaceutics.
This journal is © The Royal Society of Chemistry 2025
Please do not adjust margins

Supplementary information

Sometimes less is more: Avidity-dependent transport of targeted polymersomes across the blood-brain-barrier

Ana Alves[a,b,c], Peter Pfeifer[d], Andreia Marinho[e], Claúdia Nunes[e], Salette Reis[e], Domingos Ferreira[a,c], Marta Correia-da-Silva[b,f], Paulo C. Costa[a,c], Giuseppe Battaglia[d,g], Íris L. Batalha[*d,h], Cátia D. F. Lopes[*d]

[a] *UCIBIO – Applied Molecular Biosciences Unit, MedTech-Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, University of Porto, Rua Viterbo Ferreira, 228, 4050-313 Porto, Portugal*
[b] *Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de farmácia da Universidade do Porto, Rua Viterbo Ferreira, 228, 4050-313 Porto, Portugal*
[c] *Associate Laboratory i4HB - Institute for Health and Bioeconomy, Faculty of Pharmacy, University of Porto, Rua Viterbo Ferreira, 228, 4050-313 Porto, Portugal*
[d] *Institute for Bioengineering of Catalonia (IBEC), Barcelona Institute of Science and Technology (BIST), Carrer Baldiri Reixac, 10-12, 08028 Barcelona, Spain.*
[e] *LAQV/REQUIMTE, Departamento de Ciências Químicas, Faculdade de farmácia da Universidade do Porto, Rua Viterbo Ferreira, 228, 4050-313 Porto, Portugal*
[f] *CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, Marine Natural Products and Medicinal Chemistry Group, Avenida General Norton de Matos Matosinhos 4450-208, Matosinhos, Portugal.*
[g] *Catalan Institution for Research and Advanced Studies, Passeig de Lluís Companys, Barcelona, Spain*
[h] *Department of Life Sciences, University of Bath, Claverton Down, Bath BA2 7AY, UK*
**Correspondence: icdlb20@bath.ac.uk; clopes@ibecbarcelona.eu*

Supplementary materials and methods

Materials and solvents

All reagents and anhydrous solvents were obtained from Sigma Aldrich (Spain), except for the alpha-methoxy-omega-hydroxy poly(ethylene glycol) (methoxy-PEG; MeO-PEG-OH 2kDa) and alpha-azido-omega-hydroxy icosa(ethylene glycol) (azide PEG; N3-PEG(20)-OH) macroinitiators, which were sourced from Iris Biotech (Germany), and the gel permeation chromatography (GPC)-grade tetrahydrofuran (THF), which was obtained from Scharlab. The dialysis membranes were purchased from Fisher Scientific. All water was ultrapure.

Polymer synthesis and characterisation

PEG_{45}-*b*-PLA_{106} and N3-PEG_{20}-*b*-PLA_{106} copolymers were synthesised via 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)-mediated ring-opening polymerisation (ROP) and characterised by gel permeation chromatography and nuclear magnetic resonance spectroscopy.

D,L-lactide was dissolved in ethyl acetate at 77°C to form a saturated solution. The solution was allowed to cool firstly to room temperature and then 4°C, after which the lactide crystals were filtered under vacuum, washed with hexane, and dried overnight in a vacuum oven. The polylactic acid (PLA) block was then synthesised via DBU-mediated ROP using the corresponding polyethylene glycol (PEG) macroinitiators (MeO-PEG-OH 2kDa for pristine or N3-PEG(20)-OH for N3-conjugated PEG-*b*-PLA). The reaction scheme is illustrated in **Figure S1**.

In brief, 1g of PEG macroinitiator was introduced into a flame-dried Schlenk flask under argon flow and subsequently dried at 60°C under high vacuum for 4 hours. The flask was then refilled with argon and cooled to room temperature. Subsequently, 50 molar equivalents of recrystallised cyclic lactide monomer were added under a protective argon atmosphere, and anhydrous dichloromethane (DCM) was introduced to maintain the lactide concentration at 0.1 g mL^{-1}. Once the reagents were completely dissolved, one mol% of DBU catalyst (relative to lactide) pre-diluted in a small volume of DCM was added to the reaction. After stirring for one hour, the reaction was quenched by adding benzoic acid (5 molar equivalents relative to the catalyst). The reaction mixture was then dialysed using a 3.5 kDa cellulose ester dialysis membrane (CelluSep®, France) against 1:1 (v/v) dimethylformamide (DMF):water, with three solvent changes, followed by a dialysis against water to remove the DMF. Finally, the dispersion was freeze-dried to yield a white, glassy powder.

Gel Permeation Chromatography

Approximately 5 mg of the polymer sample were solubilised in 1 mL of GPC-grade THF and filtered using a 0.1 µm pore-size syringe filter before analysis. Samples were run at 30°C with a 1 mL min^{-1} flow rate using THF as the eluent. The experiments were performed on an Agilent 1260 Infinity II instrument equipped with a PLgel 5 µm MIXED-D 300 × 7.5 mm column, a dual angle light scattering detector, a refractive index detector, and a viscometer. Calibration was carried out using Agilent EasiVial Polystyrene

Please do not adjust margins

Please do not adjust margins

Calibration standards. Data analysis employed the triple detection method to determine the number average molecular weight (Mn), weight average molecular weight (Mw), and polydispersity index (PDI) (**Figure S2**).

Nuclear Magnetic Resonance (NMR) spectroscopy

For sample preparation, 10 mg of the polymer was solubilised in 0.75 mL of 99.8% deuterated chloroform containing 1% (v/v) tetramethylsilane (TMS). NMR spectra were acquired on a Bruker AVANCE NEO 400 (400 MHz) NMR spectrometer, with chemical shifts reported in parts per million (ppm) (**Figure S3**). Each spectrum was acquired through 32 scans.

T7 conjugation

The T7 peptide with a propargylglycine on the N-terminal (Pra-HAIYPRH) was synthesised by Genscript (Netherlands). Conjugation to N3-PEG_{20}-*b*-PLA_{106} polymer was achieved via a copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) reaction. Briefly, a 1.3 molar equivalent of Pra-T7 peptide dissolved in ultrapure water was added to a solution of N3-PEG_{20}-*b*-PLA_{106} polymer (1 molar equivalent), sodium ascorbate (5 molar equivalents), and copper(II) sulfate pentahydrate (1 molar equivalent) in a 2:1 (v/v) DMF: ultrapure water mixture. The reaction mixture was degassed with nitrogen for 30 minutes and allowed to proceed for 72 hours at room temperature with gentle stirring. The crude reaction mixture was dialysed against DMF for 1 hour using a 3.5 kDa cellulose ester dialysis membrane (CelluSep®, France) to remove low molecular weight impurities. Subsequently, the dialysed solution was purified by extensive dialysis against ultrapure water for 3 days with frequent solvent changes. The purified T7-PEG_{20}-*b*-PLA_{106} conjugate was obtained as a solid powder by lyophilisation.

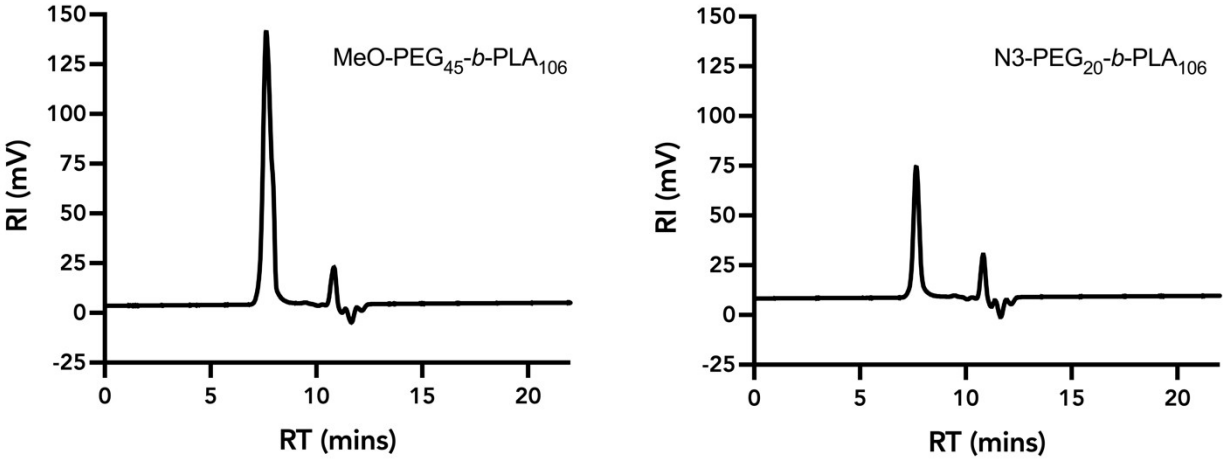
The conjugation efficiency was determined after acidic hydrolysis, followed by derivatisation with the AccQ-Fluor™ reagent kit from Waters. In brief, T7 and T7-PEG_{20}-*b*-PLA_{106} conjugate were dissolved in hydrolysis solution (trifluoroacetic acid (TFA) and concentrated hydrochloric acid (HCl, 37%) in a 1:2 (v/v) ratio) to achieve a concentration of 1mg mL^{-1}. Subsequently, 40 µL of this solution was further diluted to 0.05 mg mL^{-1}, and 600 µL was transferred into a hydrolysis tube. It was evacuated three times and purged with nitrogen. Finally, the tube was heated to 166°C for about 50 minutes in an oil bath. After cooling, the solvent was removed under reduced pressure. The hydrolysed pellet was reconstituted in 900 µL of 0.1 M HCl and derivatised following the manufacturer's instructions. The derivatised samples were finally analysed by high-performance liquid chromatography using an AccQ-Tag Ultra C18, 1.7µm (2.1 × 100 mm) column (Waters, USA). The mobile phase consisted of 0.236 M sodium acetate buffer solution with 0.017 M triethylamine at pH 5.05 (Solvent A) and 70% (v/v) Acetonitrile in water (Solvent B), with gradient elution from 100% to 66% Solvent A over 33 minutes, followed by a 5-minute at 100% Solvent B, and a 10-minute re-equilibration to 100% Solvent A. The sample was injected at a flow rate of 1.0 mL min^{-1}, with the column at 37°C. Detection was performed with excitation at 250 nm and emission at 395 nm. The conjugation efficiency was determined by quantifying the T7 peptide content in the conjugated sample (i.e., T7-PEG_{20}-*b*-PLA_{106}) using the T7 peptide calibration curve.

Supplementary Figures

Figure S1. Synthesis of PEG-*b*-PLA via DBU-mediated ring opening polymerisation.

Please do not adjust margins

Please do not adjust margins



MW averages			
Polymer	**Mn (g mol^{-1})**	**Mw (g mol^{-1})**	**PDI**
MeO-PEG$_{45}$-b-PLA$_{106}$	9926	10003	1
N3-PEG$_{20}$-b-PLA$_{106}$	8149	8254	1

Figure S2. GPC chromatogram of the pristine methoxy-PEG$_{45}$-*b*-PLA$_{106}$ and azide-PEG$_{20}$-*b*-PLA$_{106}$ block copolymers. Data shows the complete polymerisation of the PLA block with a molecular weight in the expected range and a polydispersity index (PDI) indicative of a uniform size distribution of the polymeric chains.

Please do not adjust margins

Please do not adjust margins

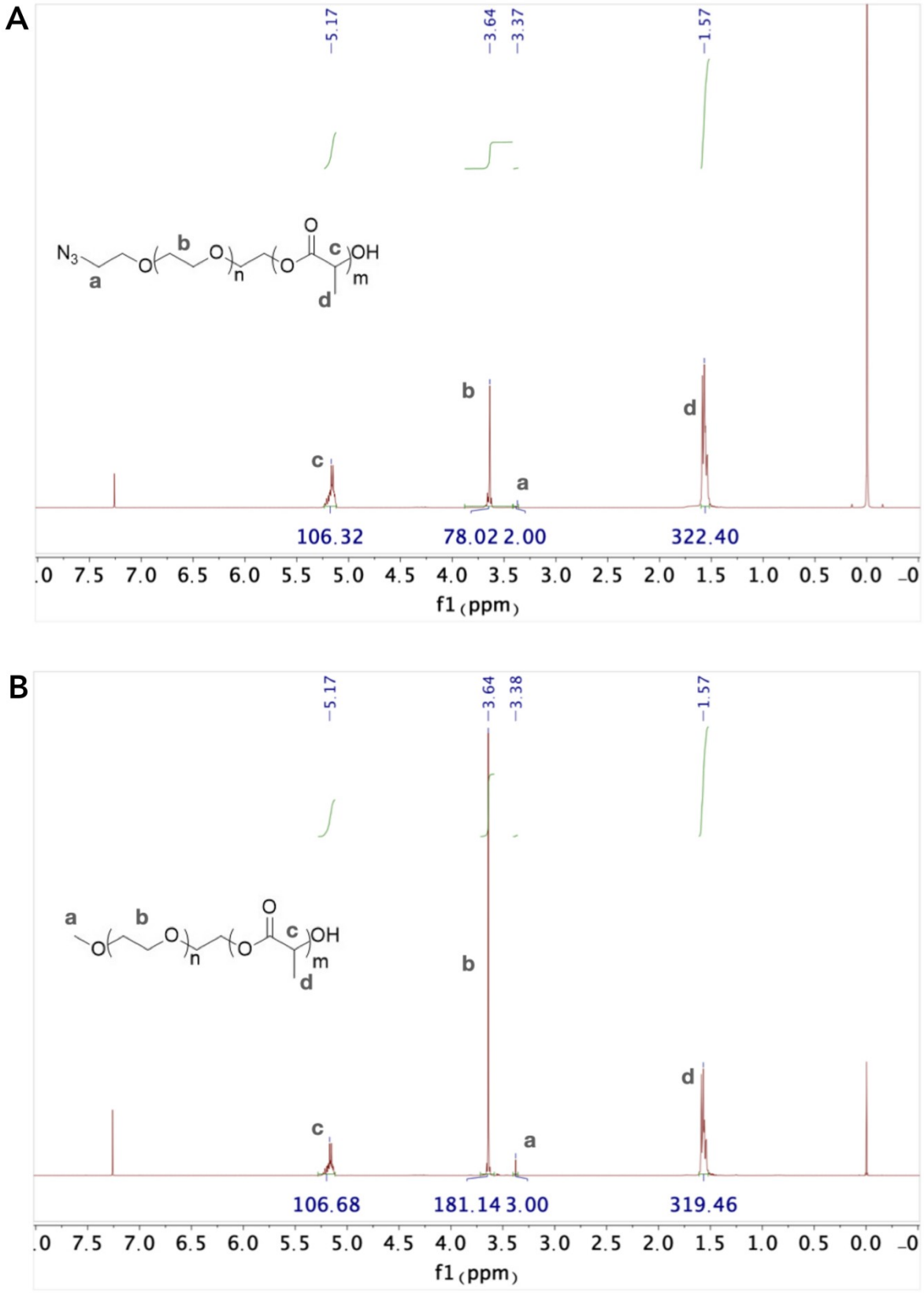


Figure S3. ^{1}H NMR spectra of the azide (A) and methoxy (B) PEG-*b*-PLA block copolymers obtained in deuterated chloroform. The methylene (-CH2) proton signals at the vicinity of the azide group (a) of PEG appear at 3.37 ppm as a broad triplet, while in the chain methylene (-CH2) proton signals (b) of this unit can be observed at 3.64 ppm. The PLA block shows -CH signals (c) at 5.17 ppm and methyl (-CH3) signals (d) at 1.57 ppm, respectively.

Please do not adjust margins