The synthesis of peptide-conjugated poly(2-ethyl-2-oxazoline)-*b*-poly(L-lactide) (PEtOx-*b*-PLA) polymeric systems through the combination of controlled polymerization techniques and click reactions

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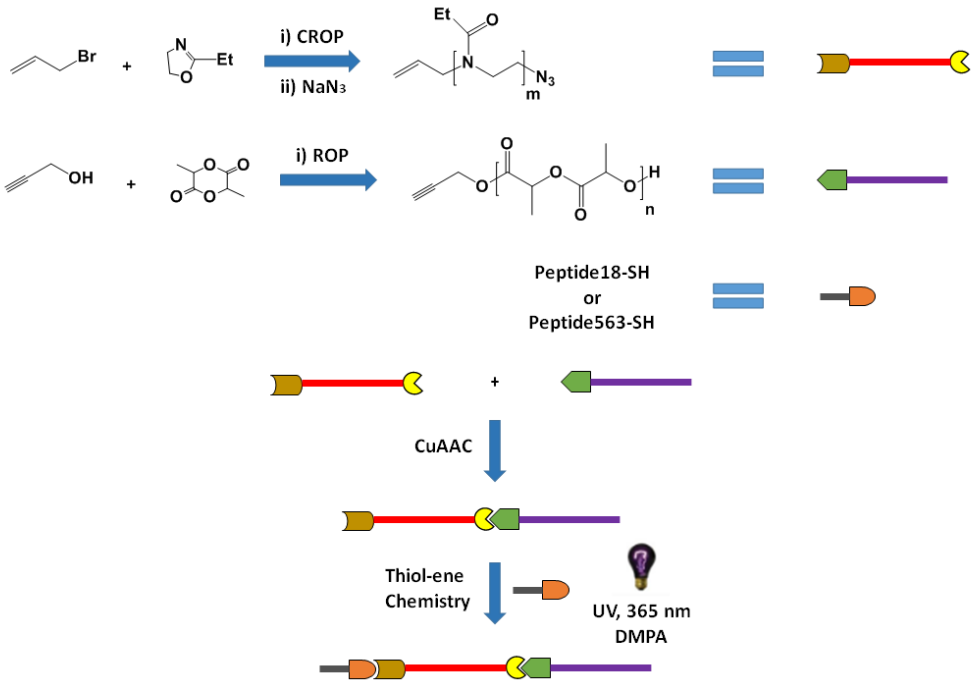
Abstract

To optimize the therapeutic effect of pharmaceutical agents, drug delivery systems tailored from FDA-approved polymers like poly(L-lactide) (PLA) is an effective strategy. Because of their hydrophobic character, these systems greatly suffer from reduced circulation time thus, amphiphilic block copolymers became favourable to overcome this limitation. Of them, poly(oxazoline)-b-poly(L-lactide) are of choice as poly(oxazoline) (PEtOx) is compatibile, biodegradable, while exhibiting minimum cytotoxicity. To tailor selective drug targeting drug delivery systems, whereby their selectivity for tumour tissues is maximised, these polymers should be decorated with so-called tumour-homing agents, such as antibodies, peptides and so forth. To this respect, we designed a new block copolymer, allyl-poly(2-ethyl-2-oxazoline)-*b*-poly(L-lactide) allyl-(PEtOx-*b*-PLA) and its subsequent conjugation to tumour-homing peptides, peptide-18 and peptide-563 at the terminal position. In this manuscript, we report our synthetic route to obtain this building block and its conjugation to tumour-homing agents.

Keywords: Poly(oxazoline), poly(l-lactide), amphiphilic block copolymer, copper-catalysed azide-alkyne cycloaddition, thiol-ene click chemistry, peptide grafting.



1. Introduction

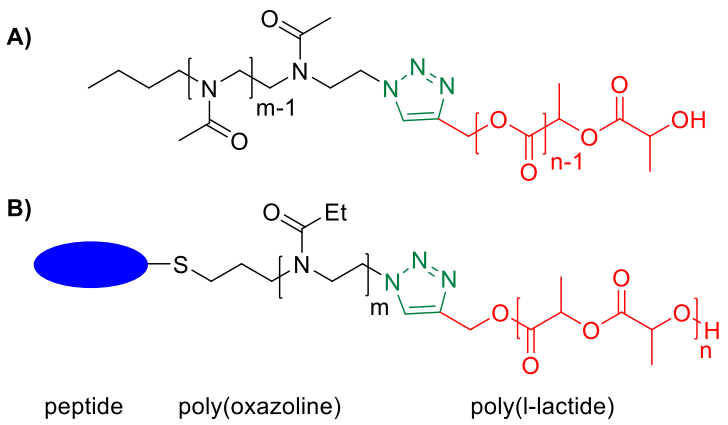
Drug delivery is a contemporary process, wherein pharmaceutical agents are administered through carrier systems to maximize their therapeutic effect [1]. In designing polymer-based systems, FDA-approved poly(l-lactide) (PLA) is a common ingredient because of their high biocompatibility [2]. In this context, it is worth noting that the hydrophobic nature of PLA posed a problem as it augments the uptake of drug-loaded nanoparticles (NPs) through mononuclear phagocyte system, resulting in diminished therapeutic efficiency [3]. Thus, many research groups explored the notion of decorating the surface of PLA with hydrophilic polymers, which resulted in amphiphilic block-co-polymers, such as PLA-grafted dextran [4], PLA-*b*-poly(2-methacryloyloxyethylphosphorylcholine) [5], PLA-*b*-poly(N,N-dimethylaminoethyl methacrylate) [6], PEG-*b*-PLA [7], poly(oxazoline)-*b*-PLA [8-9] and so forth.

Amongst these building blocks, the latter stands out on the grounds that poly(oxazoline) is highly biocompatible, biodegradable, and favourably immunogenicity, whilst exhibiting minimum *in vivo* cytotoxicity [10-17]. Furthermore, poly(oxazoline) present “stealth” behaviour whereby they shield the nanocarriers from the immune system [17]. From the standpoint of synthetic chemistry, the fashionable cationic ring-opening polymerization (CROP) of 2-substituted-2-oxazolines yields well-defined block copolymers in one pot, through the sequential polymerisation in tandem with

"one-shot" copolymerization techniques to afford polymer chains in many forms; random, quasi-diblock, and block copolymers [18-20]. Also, they are structurally very versatile, in that functional groups could be introduced either to the terminal positions or the backbone, in a controlled manner. It is needless to say that this control provides freedom for an orthogonal conjugation of biomolecules (such as drugs, fluorescent labels, targeting agents and so forth).

As alluring as they appear, the emergence of these polymeric materials did not obviate all the problems in the related field. Surely, they are the right direction in terms of drug delivery, however; their efficiency as the drug-carrier is still questioned in part. This is because nanoparticle-based drug delivery systems fail to utterly distinguish unhealthy cells over normal ones, resulting in drug side-effects. Therein, a judicious solution is to decorate these nanoparticles with biological molecules (such as peptides [21-26], proteins [27-28], aptamers [29-30], and antibodies [31-32]), which direct these materials to a precise destination. Otherwise known as "tumour-homing" agents in chemotherapy, these molecules are reported to ameliorate the efficiency of these nanoparticles in transporting therapeutics by recognising to receptor sites on tumours [33]. To benefit from this notion, the synthetic route to PEG-*b*-PLA block copolymers should be crafted in a way that it permits grafting tumour-homing agents.

Within this scope, we designed two poly(2-ethyl-2-oxazoline)-*b*-poly(L-lactide) block copolymers (**1a**, and **1b**), which harbour tumour-homing peptide (**Scheme 1**). The strategy we devised to access these materials relies on the independent synthesis of allyl-poly(2-ethyl-2-oxazoline)-N_3 and alkyne-poly(L-lactide) blocks, followed by their assembly through "Click" chemistry. To tailor targeted drug delivery systems from these building blocks, we have then investigated the grafting of two tumour-homing peptides, **peptide-18** and **peptide-563** to obtain **peptide18/peptide563-poly(2-ethyl-2-oxazoline)-*block*-poly(L-lactide)** conjugates **1a** and **1b**. As in our previous work [34], functional groups on PEtOx block govern orthogonality in this approach; as such that azide group allows tethering PLA block at first through CuAAC click reaction [35] whereas allyl group paves the way for conjugating peptides via thiol-ene click reaction [36]. In this manuscript, we present our methodology to access **1a** and **1b** and discuss experimental details, regarding the grafting of two different peptides on these molecules.



Peptide18-PEtOx-*b*-PLA 1a
Peptide563-PEtOx-*b*-PLA 1b

Scheme 1. A) Poly(oxazoline)-*b*-PLA block copolymer reported in the literature [8] and **B)** Novel peptide-PEtOx-*b*-PLA conjugates **1a** and **1b** reported in this manuscript.

2. Experimental

2.1. Materials

All l-amino acids which are protected with Fmoc, Wang resin (4-Benzyloxybenzyl alcohol resin), (100-200 mesh, 0.85 meq/g), hydroxybenzotriazole (HOBt), diisopropylcarbodiimide (DIC) and 4-dimethylaminopyridine (DMAP) were purchased from Chem-Impex (USA). *N*,*N*-dimethyl formamide (DMF), diethyl ether, methanol (MeOH), dichloromethane (DCM) and acetonitrile (ACN) were obtained from Merck (Germany). DCM was further distilled over calcium hydride under nitrogen. Double-deionized water was prepared with Water Purification Systems (Milli-Q® Advantage A10® (TOC: 7 ppb, 18.2 MΩ.cm at 25 ºC)).

L-lactide (Aldrich, Netherlands) was recrystallised from tetrahydrofuran (THF, VWR, EC). 2-ethyl-2-oxazoline (EtOx, Aldrich) was dried over calcium hydride (Aldrich), distilled under vacuum, and stored under an inert atmosphere, before use. Allyl bromide (reagent grade, Aldrich) and propargyl alcohol (PA, Acros) were purified via vacuum distillation. Toluene (Aldrich) and acetonitrile (ACN, J. T. Baker) were distilled from calcium hydride, before use. Sodium azide (NaN_3, Aldrich), stannous octoate ($Sn(Oct)_2$) (Aldrich), 2,2-bis(hydroxymethyl)propionic acid (DMPA, Aldrich), all other reagents and solvents were directly used. Dialysis tubings with specified molecular weight cut-off and closures were purchased from Spectrumlabs.

2.2. Characterisations

The crude materials of peptides are purified through a Dionex UltiMate 3000 reverse-phase HPLC system, equipped with a Thermo Scientific Hypersil Gold C18 column (250 x 10 mm, particle size 5 μm), using a gradient of acetonitrile/water with 0.1% trifluoroacetic acid (v/v) (5-100%, 1-70 min) and a flow rate of 2 mL/min at 40 ºC. The crude material was dissolved in double-deionized water to a concentration of approximately 20 mg/mL. In each batch, up to 30 mg crude peptide was applied to HPLC and the purity of collected fractions was assessed by analytical RP-HPLC.

Analytical HPLC spectra of peptides were recorded on a Dionex UltiMate 3000 HPLC system equipped with a Thermo Scientific Acclaim^{TM} 120 C18 column (46 x 150 mm, particle size 3 μm). Peptides were eluted in a gradient of acetonitrile/water with 0.1% trifluoroacetic acid (v/v) (5-100%, 1-30 min, flow rate = 0.4 mL/min) and combined fractions were lyophilised with Telstar Cryodos Freeze Dryer to yield the pure peptides.

High-resolution mass spectra (HRMS) were recorded on an Agilent 6530 Q-TOF mass spectrometer equipped with ESI (Electrospray Ionization) source. Acquired masses are reported as m/z $[M+nH]^{n+}$ and compared with calculated masses.

Fourier transform infrared (FT-IR) spectroscopy measurements were performed utilizing a Perkin Elmer Frontier spectrometer (4000-500 cm^{-1}).

Nuclear magnetic resonance measurements were recorded on a Varian 600 Spectrometer operating at 599.90 MHz. The chemical shifts were given in δ (ppm) with respect to the internal standard TMS and the coupling constants were reported in Hertz. Splitting patterns were: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad signal).

Gel permeation chromatography (GPC) measurements were carried out on an Agilent 1260 Infinity Multi-Detector GPC/SEC system with light scattering (390-MDS 15/90 LS), refractive index (Agilent 1260 Infinity MDS RID), and viscometer (390-MDS) detectors with 2 distinctive column systems: (i) PLgel Mixed-D column (7.5 x 300 mm^2; 5 μm) for PLA-Alkyne, as well as the final products **1a** and **1b**, (ii) PL Aquagel-OH Mixed H column (7.5 x 300 mm^2; 8 μm) for Allyl-PEtOx-N_3.

Thiol-ene click reactions were performed in a commercial ultraviolet photoreactor device (Kerman UV/Vis), which was equipped with 18 lamps (8 W) shining a UV light at 365 nm.

2.3. Peptide Synthesis

2.3.1. Loading of First Amino Acid onto Wang Resin

Wang resin (200 mg, 0.7 mmol/g) was placed in a 10 ml glass reaction vessel and was swollen in dry DCM for one hour and drained. The first amino acid was loaded to the pre-swelled resin, using 2 equivalents of amino acid, 4 equivalents of HOBt, 2 equivalents of DIC, and 2 equivalents of DMAP in DMF with a reaction time of 20 hours at RT, after which the resin was washed with DMF (3x), then DCM (3x) and air-dried. The attachment of first amino acid was checked by treating three sets of approximately 1 mg of resin with a solution of piperidine in DMF (20%, 3 ml) and suspending them for 20 min. 1 ml solution from each vial was taken into 1 ml UV quartz cuvette and the absorbance at 290 nm was measured. Experimental resin substitution was calculated with the formula, Loading = A_{290nm} /(mg of resin x 1.65). If the first amino acid was successfully loaded, the resin was re-swelled in DMF, then capped with 2 equivalents of benzoyl chloride and 3.6 equivalents of pyridine in DMF for 30 min. After washing the resin with DMF (3x), MeOH (1x), and DMF (3x), the peptide synthesis continued, as described in the following section.

2.3.2. General Solid Phase Peptide Synthesis

Peptide-18 (H-Cys-Trp-Arg-Glu-Ala-Ala-Tyr-Gln-Arg-Phe-Leu-OH), and peptide-563 (H-Cys-Gly-Arg-Phe-Leu-Thr-Gly-Gly-Thr-Gly-Arg-Leu-Leu-Arg-Ile-Ser-OH) were prepared by Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) on pre-loaded Wang resin. Peptide synthesis was carried out on CEM Discover Bio-Manual Microwave Peptide Synthesizer in the scale of 0.1 to 0.25 mmol. The dry resin was swelled with DMF before use. Generally, the sequence extension of peptides was performed with Fmoc-L-amino acids (3.0 eq.), HBTU (*N,N,N′,N′*-tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate, 2.85 eq.), and DIPEA (*N*,*N*-diisopropylethylamine, 6 eq.) in DMF *via* microwave (MW) heating (20 W, 10-15 min). Arginines were activated with HCTU (2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) in lieu of HBTU, based on the observation than these residues demand more potent coupling agent the latter. To eradicate the racemization of cysteine residues, MW heating was avoided in course of its coupling reactions. Upon each coupling reaction, unreacted sites were capped (20 W, 2 min) with a mixture of DMF/pyridine/Ac_2O (10:1:1, v/v/v). After each coupling and the following deprotection reaction, the completion of coupling reactions was assured with

Kaiser Test [37]; otherwise, double-couplings were performed. After the coupling reactions, Fmoc groups were removed with a solution of piperidine in DMF (20%, 5 ml) via MW heating (20 W, 2x3 min). Subsequent to the coupling of last amino acid and the removal of its Fmoc group, the resin was washed with DMF (3x), DCM (3x), MeOH (1x), DCM (3x) and ether (1x) respectively and then, it was air-dried for one hour. To cleave the peptide from the resin and to concurrently remove the protecting groups on side-chains, the resin was treated with a mixture of TFA (trifluoroacetic acid)/water/TIS (triisopropyl silane)/EDT (ethanedithiol) (90:5:2.5:2.5 v/v/v/v) for 3 hours. Then, peptides were precipitated from cold diethyl ether, centrifuged and the supernatant was removed; this procedure was repeated three more times as a pre-purification to remove shorter peptide chains. The purification of the crude material by RP-HPLC, followed by lyophilisation gave pure peptides, whose purities were checked through analytical RP-HPLC (**Figure S1** and **Figure S2**). Finally, the peptides were characterised by mass spectrometry (**Figure S3** and **Figure S4**).

2.4. The Synthesis of Amphiphilic Block Copolymers

2.4.1. The Synthesis of α-Allyl-ω-Azido-Poly(2-ethyl-2-oxazoline) (Allyl-PEtOx-N_3) 4 [35, 38-43]

A flask equipped with a stirring bar was dried up with a heat gun. Then, it was capped with a septum and it was once again warmed up with a heat gun under vacuum. It was subsequently cooled to room temperature under vacuum and a solution of allyl bromide **2** (123 μL, 1.42 mmol), and EtOx **3** (5 ml, 49.53 mmol) dissolved in ACN (15 mL) was added to the flask under an inert atmosphere. After polymerization at 130 ºC for 15 hours, the reaction was chilled to room temperature. Then, NaN_3 (0.37 g, 5.68 mmol) was added as a powder at once and the reaction was stirred in the dark for 1 day and at 65 ºC to terminate the reaction. The reaction mixture was, then, cooled down to room temperature and the solvent was evaporated *in vacuo*. Thereafter, the crude compound was dissolved in DCM (15 ml) and the title material was precipitated from cold diethyl ether and dried under vacuum. Yield = 4.47 g, 91%; $M_{n,theo}$ = 3200 Da, $M_{w,GPC}$ = 4000 Da, $M_{n,GPC}$ = 3600 Da, polydispersity index (PDI = $M_{w,GPC}$ / $M_{n,GPC}$) = 1.11. FT-IR: ν [cm^{-1}] 1630 (carbonyl) and 2100 (azide). ^{1}H-NMR ($CDCl_3$): δ 1.1-0.9 (3H, -N-CO-CH_2-C***H*****$_3$**), 2.4-2.2 (2H, -N-CO-C***H*****$_2$**-CH_3), 3.5-3.3 (4H, -N-C***H*****$_2$**-C***H*****$_2$**-), 3.8 (2H, CH_2=CH-C***H*****$_2$**-), 5.4 (2H, C***H*****$_2$**=CH-CH_2-), 5.8 (1H, CH_2=C***H***-CH_2-).

2.4.2. The Synthesis of α-Alkyne-Poly(L-lactide) (PLA-Alkyne) 7 [35, 39, 44]

PLA-Alkyne **7** was synthesized through ring-opening polymerization (ROP) of L-lactide in the presence of $Sn(Oct)_2$, and propargyl alcohol, as the catalyst, and the initiator, respectively. Propargyl alcohol **5** (PA, 57 μL, 0.98 mmol) and L-lactide **6** (10 g, 69.38 mmol) were added to a flask and a solution of $Sn(Oct)_2$ (19.43 μL, 0.06 mmol) in toluene (10 mL) was subsequently introduced. The reaction mixture was deaerated with nitrogen and immediately submerged in a thermostated oil bath at 120 ºC for 5 hours. Once the reaction was completed, the solvent was evaporated *in vacuo* and then, the crude material was redissolved in DCM (15 mL). The title compound was precipitated from cold methanol and dried under vacuum oven at room temperature. Yield = 8.10 g, 81%, $M_{n,theo}$ = 8300 Da, $M_{w,GPC}$ = 19300 Da, $M_{n,GPC}$ = 14400 Da, polydispersity index (PDI = $M_{w,GPC}$ / $M_{n,GPC}$) = 1.34. FT-IR: ν [cm^{-1}] 660, 750, 860, 1050, 1090, 1180, 1260, 1380, 1460, 1740, 2945, 2980, 3290. ^{1}H-NMR ($CDCl_3$): δ 1.48-1.69 (m, (CO)-CH-(C***H*****$_3$**)O on PLA), 2.50 (s, 1H, CH_2-C≡C***H***), 4.34 (m, (CO)-C***H***-(CH_3)OH end-group of PLA), 4.71 (s, 2H, C***H*****$_2$**-C≡CH), 5.16 (m, (CO)-C***H***-(CH_3)-O on PLA).

2.4.3. The Synthesis of PEtOx$_{3600}$-*b*-PLA$_{14400}$ 8 Amphiphilic Block Copolymer via Copper-Catalysed Azide-Alkyne Cycloaddition (CuAAC) Click Chemistry [35, 39, 44-46]

A flask equipped with a stirring bar was dried up *via* a heat gun under vacuum. To this flask, a solution of allyl-PEtOx-N_3 **4** (0.324 g, 0.09 mmol), PLA-alkyne **7** (1,296 g, 0.09 mmol), sodium ascorbate (0.089 g, 0.45 mmol), and copper sulfate (0.015 g, 0.09 mmol) dissolved in DMF (25 mL) was added. After the mixture was deaerated through bubbling with nitrogen, the reaction was stirred in the dark and at room temperature for one day. Then, the reaction mixture was passed through a silica column and the solvent was evaporated *in vacuo*. The final solid material was dissolved in DCM (15 ml) and the title compound was precipitated from cold methanol. The product **8** was dried under vacuum. Yield = 1.35 g, 83%, $M_{n,\,theo}$ = 11500 Da, $M_{w,\,GPC}$ = 20400 Da, $M_{n,\,GPC}$ = 16200 Da, polydispersity index (PDI = $M_{w,GPC}$ / $M_{n,GPC}$) = 1.26.

2.5. The Grafting of Targeting Agents to PEtOx$_{3600}$-*b*-PLA$_{14400}$ Amphiphilic Block Copolymer via Thiol-ene Click Chemistry

2.5.1. The Grafting of Peptide-18 to allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$ (Peptide-18-PEtOx$_{3600}$-*b*-PLA$_{14400}$) 1a [47]

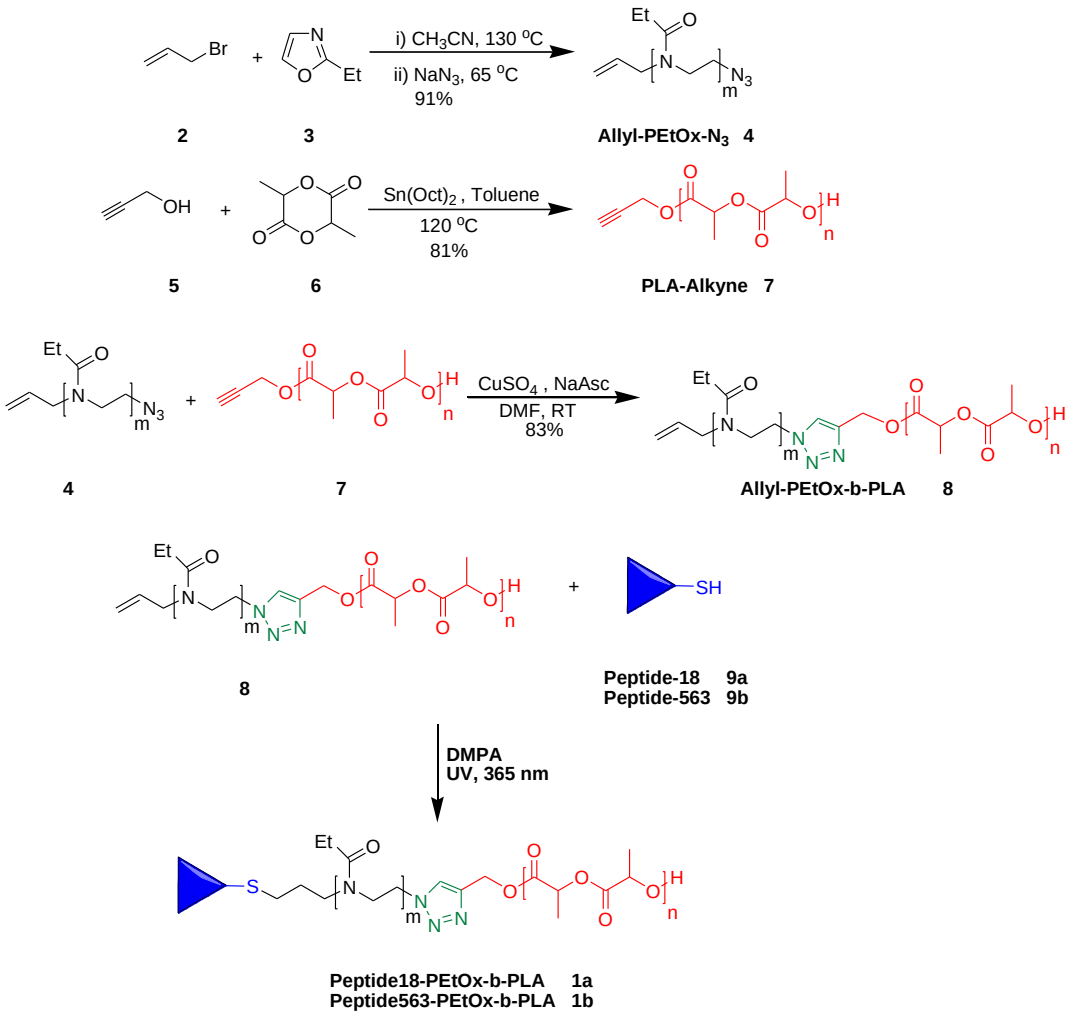
A solution of allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$ **8** (81 mg, 4.49 μmol), peptide18-SH **9a** (15 mg, 8.98 μmol), DMPA (6.03 mg, 44.91 μmol) dissolved in a mixture of DCM/MeOH (8mL/2mL) was added to a tube equipped with a stirring bar. After a process of exhausting-refilling for three times, the tube was irradiated under a 365 nm UV lamp (144 W) for one hour in a way that the distance from the lamp to the tube was approximately 15 cm. Upon the completion of the reaction time, the crude material was precipitated from cold methanol. Then, the precipitate was dissolved in DCM and dialysed overnight against deionized water (molecular weight cut-off = 2000 Da) to remove unreacted peptide-18 and the final product was obtained through lyophilisation.

2.5.2. The Grafting of Peptide-563 to allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$ (Peptide-563-PEtOx$_{3600}$-*b*-PLA$_{14400}$) 1b [47]

A solution of allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$ **8** (84 mg, 4.63 μmol), peptide563-SH **9b** (20 mg, 9.25 μmol), DMPA (6.20 mg, 46.24 μmol) dissolved in a mixture of DCM/MeOH (8mL/2mL) was added to a tube having a stirring bar. After a process of exhausting-refilling for three times, the tube was irradiated under a 365 nm UV lamp (144 W) for one hour in a way that the distance from the lamp to the tube was approximately 15 cm. Then, the crude material was precipitated from cold methanol. The precipitate was dissolved in DCM and dialyzed overnight against deionized water (MWCO = 2000 Da) to remove unreacted peptide-563 and the final product was obtained with lyophilisation.

3. Results and Discussion

Depicted in **Scheme 2**, our synthetic strategy to acquire peptide-grafted **1a-1b** commences with the synthesis of allyl-PEtOx$_{3600}$-N$_3$ **4** through the living CROP of EtOx **3**, which is initiated by allyl bromide **2** and terminated with sodium azide [38]. Then, the polymerization of L-lactide **6** was carried out through the ROP, which was initiated by propargyl alcohol **5** [35, 39, 44]. Subsequently, the amphiphilic block copolymer allyl ended-PEtOx$_{3600}$-*b*-PLA$_{14400}$ **8** was synthesized by CuAAC click chemistry of **4** and **7** that was catalysed by copper sulfate. In the final step, peptide-18 or peptide-563 is grafted on **8** through well-established photoinduced thiol-ene click chemistry to give **1a** or **1b**, respectively [47].



Scheme 2. The synthetic strategy to obtain peptide-conjugated PEtOx-*b*-PLA.

As outlined in the previous section, the polymerization reaction to obtain **4** was initiated with allyl bromide ([monomer]/[initiator] ratio = 35:1) and was terminated with sodium azide. In ^{1}H-NMR, the peaks at 3.3-3.5, 2.2-2.4 and 0.9-1.1, which were assigned to the protons of ethylene moiety (4H, -N-C***H*****$_2$**-C***H*****$_2$**-N) on the repeating units, methylene (2H, CH_3-C***H*****$_2$**-C=O-), and methyl (3H, C***H*****$_3$**-CH_2-C=O-) groups (**Figure 1a**), supports the formation of **4**. Furthermore, the peaks at 5.8 and 5.4 were assigned to allyl (1H, CH_2=C***H***-CH_2-) and (2H, C***H*****$_2$**=CH-CH_2-), respectively and they support the presence of allyl group on **4** [46-49]. In FT-IR of 4 **(Figure 1b)**, the stretching of allyl group falls in overcrowded 1600-1700 cm^{-1} region, thus; FT-IR spectrum of **4** fails to confirm the presence of allyl group. As for the azide group, the antisymmetric stretching vibration band of PEtOx-N_3 was observed at 2100 cm^{-1} in the FT-IR spectrum, which is in line with the literature **(Figure 1b)**. In overall, these results are a firm indication that compound **4** was synthesized

successfully and that it was capped with allyl and azide groups, as expected. It is also worth noting that the corresponding M_n (number-average molecular weight) value of allyl-PEtOx-N_3 was determined by GPC and found to be 3600 Da, with a polydispersity index (PDI) of 1.11 (**Figure S5**).

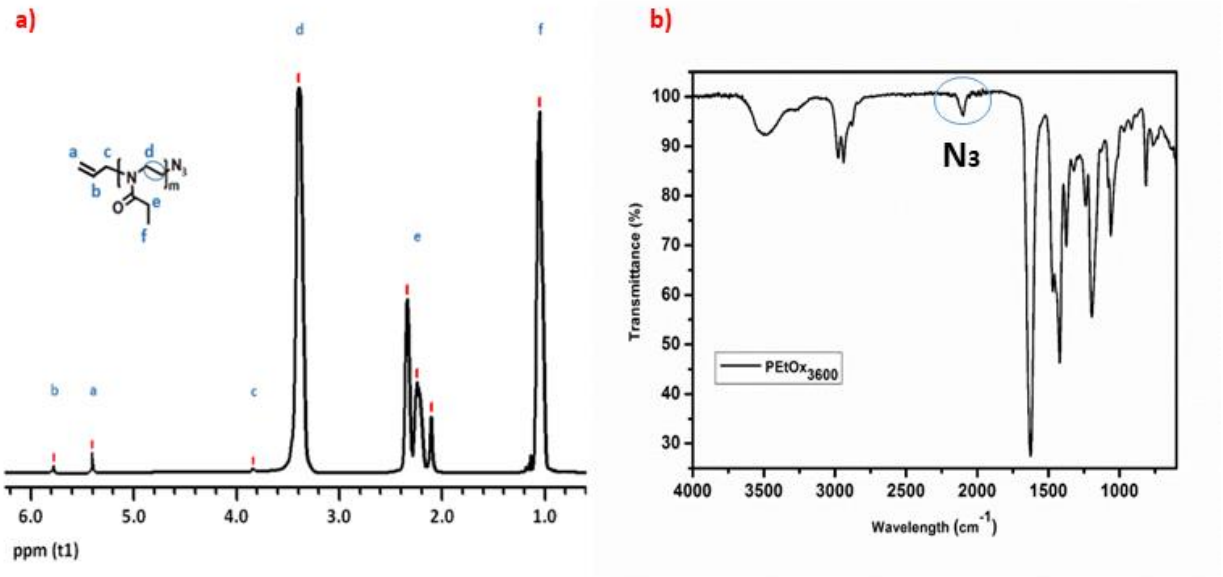


Figure 1. a) The ^{1}H-NMR spectrum of PEtOx$_{3600}$. **b)** The FT-IR spectrum of PEtOx$_{3600}$.

Subsequently, PLA-alkyne **7** was obtained through the coordination-insertion ROP of L-lactide **6**, which was initiated by propargyl alcohol. Following the polymerization reaction, the structure of **7** was elucidated with ^{1}H-NMR, in which the methine ((C=O)-C***H***-CH_3) and methyl ((C=O)-CH-C***H$_3$***) protons of PLA were observed at *ca.* 5.2-5.6, and 1.5-1.7 ppm, whereas the methylene (C***H$_2$***-C≡CH) and methine (CH_2-C≡C***H***) protons of propargyl group were detected at 4.7-4.8, and 2.5-2.6 ppm, respectively. Its structure was further confirmed by FT-IR spectroscopy; the alkyne and ester groups were determined at 3290 (C≡C-H unit), 2090 (C≡C unit), 1740 (C=O unit) and 1090 (C-O-C=O unit) cm^{-1} **(Figure 2a)**. As for the size of PLA-alkyne, the M_n and PDI values were found to be 14400 Da, and 1.34 through GPC analysis **(Figure 2b)** [47, 48].

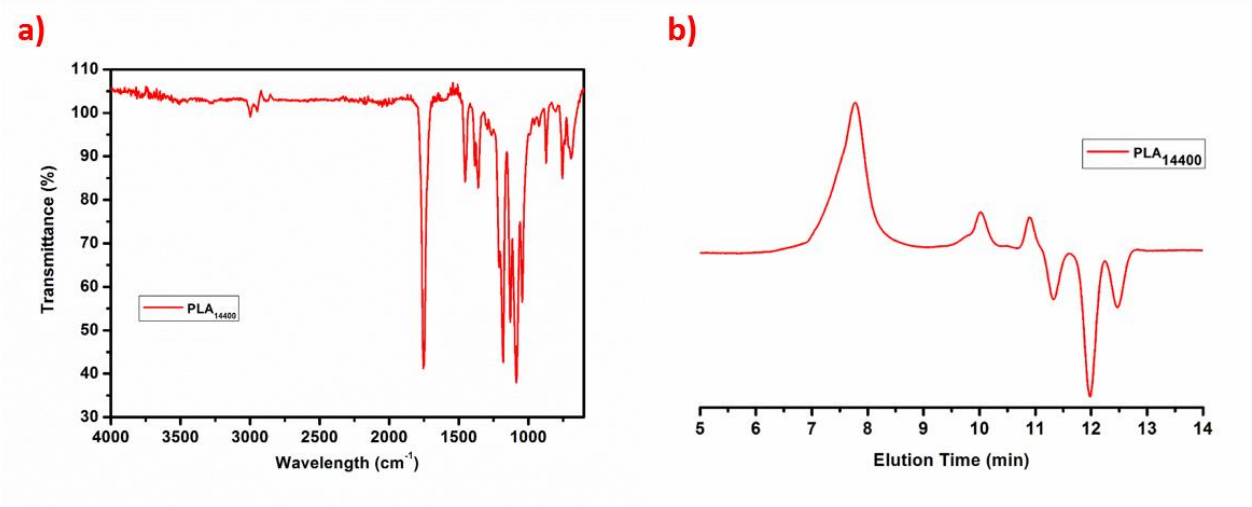


Figure 2. a) The FT-IR spectrum of PLA_{14400}. **b)** The GPC chromatogram of PLA_{14400}.

Next, the amphiphilic block copolymer allyl-$PEtOx_{3600}$-*b*-PLA_{14400} **8** was synthesized through CuAAC click reaction of allyl-PEtOx-N_3 **4** with PLA-alkyne **7**. The product **8** was characterised through the ^{1}H-NMR, wherein the distinctive protons of both **4** and **7**, with the methylene (C***H*****$_2$**-C≡CH) protons of **7** shifted to 5.2 ppm, were assigned in full **(Figure 3)**. Yet, more compelling evidence, regarding the structure of **8**, is the proton of the triazole ring at *ca.* 8.0 ppm, which markedly substantiates the tethering of both blocks through Click reaction. In regards to its structural analysis, FT-IR spectrum of allyl-$PEtOx_{3600}$-*b*-PLA_{14400} **8** also supports ^{1}H-NMR spectrum; the distinctive band of azide group on **4** at 2100 cm^{-1}, in conjunction with the bands of alkyne on **7** at 2090 and 3290 cm^{-1}, disappeared as new bands attributed to the carbonyl, and (C-O-C=O) groups on **6** are observed at 1740 and 1090 cm^{-1}, respectively. Other functional groups on **8** were thoroughly characterised upon comparing its FT-IR spectrum with that of **4** and **7**, as given in **(Figure 4)**.

The stoichiometry between two blocks was determined to be 1:1 through the ratio of methyl protons (f) of PEtOx and that of the methyl ((C=O)-CH-C***H*****$_3$**) protons (l+n) of PLA. Similarly, the $M_{n,NMR}$ of the block copolymer is calculated through the integral ratio of the triazole proton (i) with that of the methyl protons (l+n) of PLA **(Figure 3)**. Also, the GPC chromatograms of initial precursors **4** and **7** displayed unimodal patterns with narrow molecular weight distributions. Therein, GPC chromatogram of allyl-$PEtOx_{3600}$-*b*-PLA_{14400} **8** appeared to be monomodal and distinctly moved towards higher molecular weight regions. Interestingly, block copolymer **8** has a lower polydispersity index than does PLA, which takes to mean that the conjugation of allyl-PEtOx-N_3

to PLA improved molecular weight distribution of **8** throughout CuAAC click reaction **(Figure 5)**. To conclude, the structural characterisations of this key intermediate through ^{1}H-NMR, in tandem with FT-IR, and GPC denoted that the synthetic route depicted in **Scheme 2** yielded allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$ **8** and that its successful synthesis was achieved by CuAAC click reaction under relatively mild condition [35, 39].

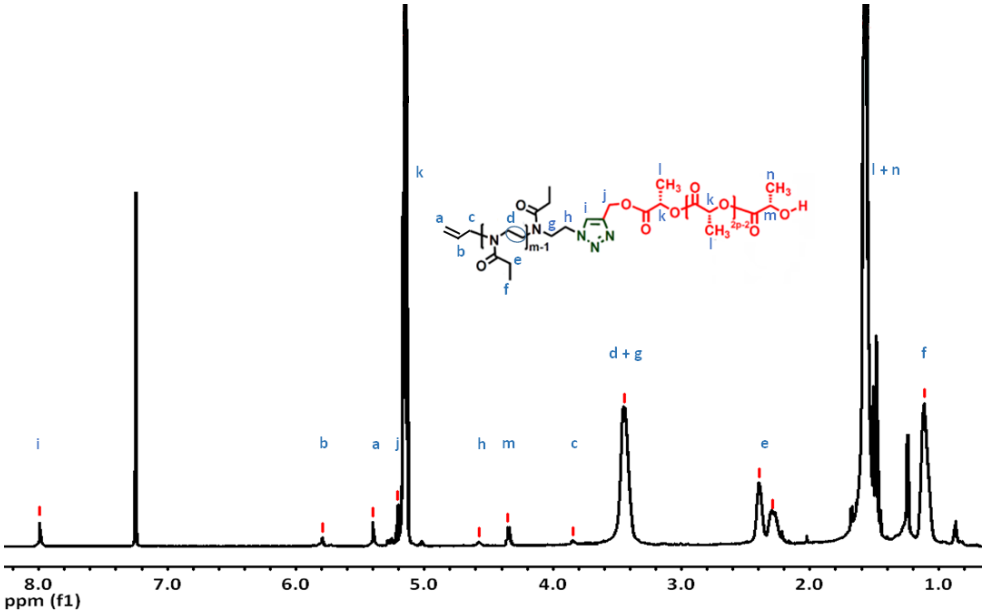


Figure 3. The ^{1}H-NMR spectrum of allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$.

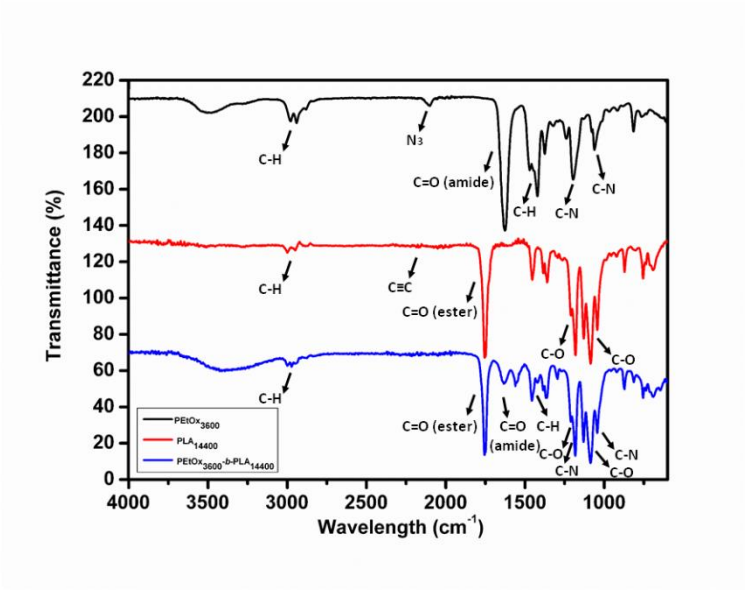


Figure 4. The FT-IR spectrum of allyl-PEtOx$_{3600}$ and allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$.

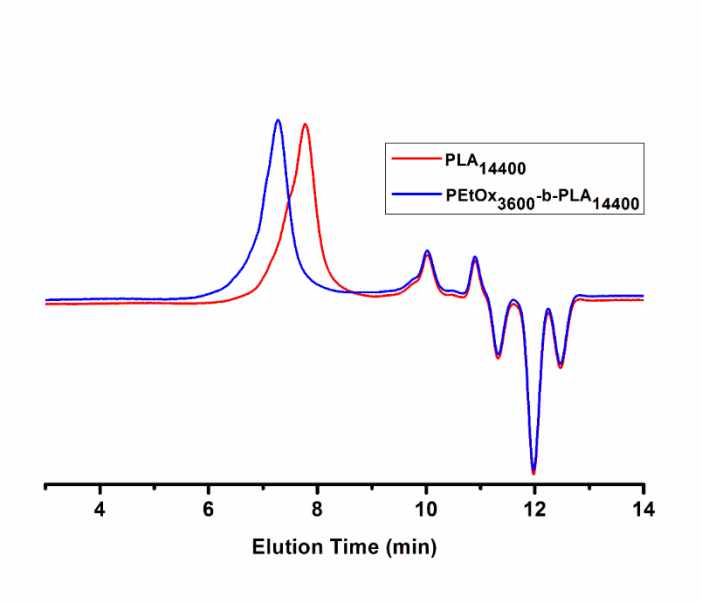


Figure 5. The GPC chromatogram of allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$.

As proof of orthogonality in our synthetic approach, we explored the grafting of peptide-18 **1a** [48, 49] and peptide-563 **1b** [50] to allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$ **8**, respectively. Recognized as tumour-homing agents, these peptides offer a platform to direct chemotherapeutics predominantly to tumour tissues, especially when antibodies perform rather poorly in this capacity [48]. Inspired from this notion, we have envisioned that grafting **9a** or **9b** to allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$ may be instrumental in designing novel polymeric materials, such as **1a** and **1b**, which will be conducive to engineering polymeric drug delivery systems.

With this rationale in mind, we have synthesized **1a** and **1b** from allyl-PEtOx$_{3600}$-*b*-PLA$_{14400}$ **8** and peptide-18/peptide-563, respectively, and purified with dialysis, as detailed in the experimental section. In ^{1}H-NMR of **1a (Figure 6)**, aromatic protons of Tyr, Phe, and Trp residues, which were central to the characterization of the products, are observed at 6.62 ppm, 6.95 ppm, 7.22-7.06, and 7.48-7.31 ppm. Additionally, the peaks, corresponding to the aromatic protons of Phe residues, were observed at 7.41-7.12 ppm in ^{1}H-NMR of **1b (Figure 7)** [51]. Besides, the formation of PLA-block was supported by the characteristic protons of (l+n), (m) and (k) at 1.42-1.65, 4.38, and 5.19 ppm. On the other hand, PEtOx blocks in both **1a** and **1b** are characterised by the multiple peaks at 3.3-3.5 ppm, 2.2-2.4, and 0.9-1.1 ppm which were assigned to ethylene (-N-C$\boldsymbol{H_2}$-C$\boldsymbol{H_2}$-N), methylene (CH_3-C$\boldsymbol{H_2}$-C=O-), and methyl (C$\boldsymbol{H_3}$-CH_2-C=O-) protons [35].

The grafting of peptides tumour-homing peptides onto **8** to give **1a** and **1b** was also confirmed by GPC analysis; after the conjugation of the peptides, the GPC traces of peptide18-PEtOx$_{3600}$-*b*-PLA$_{14400}$ and peptide563-PEtOx$_{3600}$-*b*-PLA$_{14400}$ were rather monomodal and more crucially, these traces were considerably shifted to higher molecular weight regions **(Figure 8)**. Quite naturally, the increase in M_n of peptide563-PEtOx$_{3600}$-*b*-PLA$_{14400,}$ was more substantial than that of its counterpart **1b**, merely because peptide-563 has higher molecular weight than does its counterpart, peptide-18. As regards to our overall goal, these results reported hereby are expounded as peptide-SH and PLA-alkyne **7** being orthogonally sequentially grafted onto allyl-PEtOx-N_3 **4**, as originally devised in **Scheme 2**.

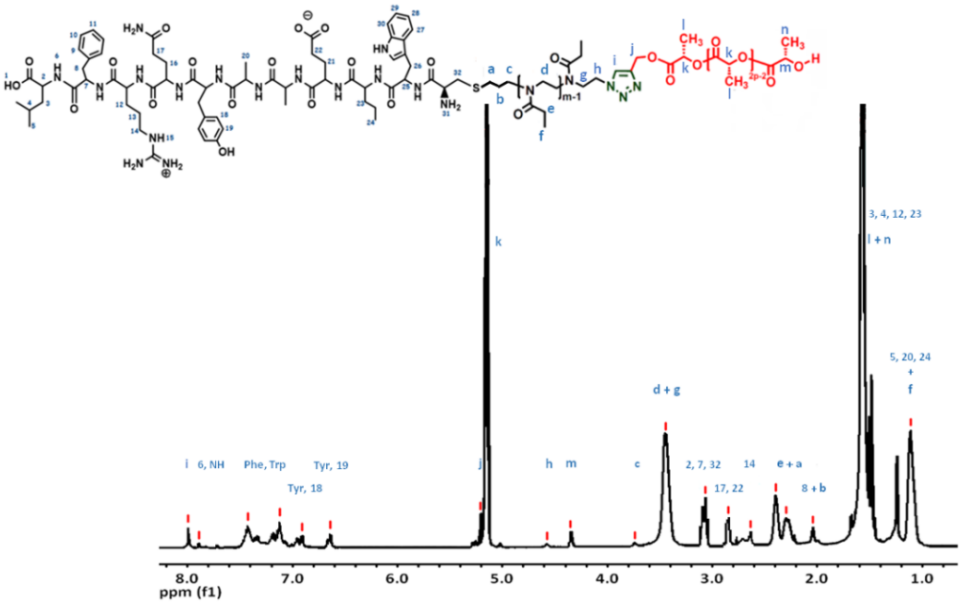


Figure 6. The ^{1}H-NMR spectrum of peptide18-PEtOx$_{3600}$-*b*-PLA$_{14400}$.

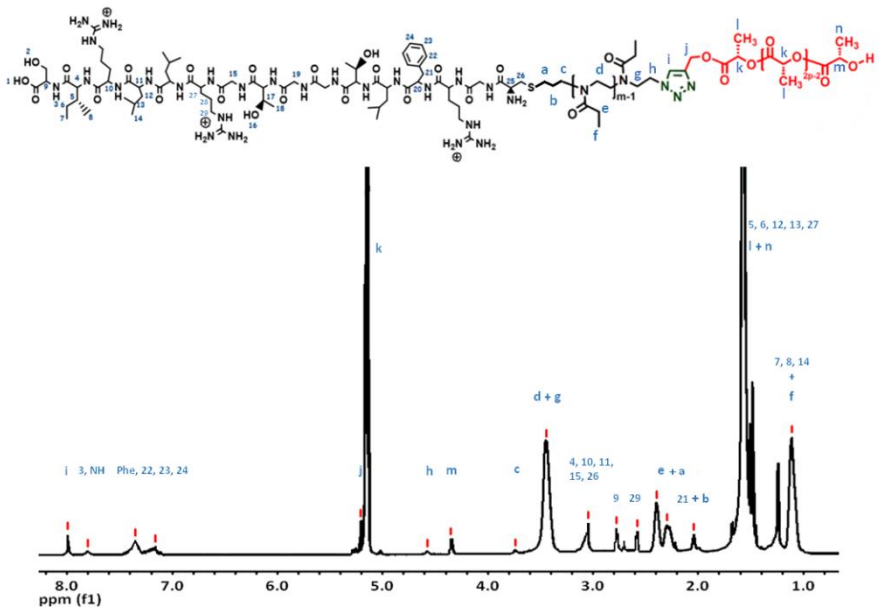


Figure 7. The ^{1}H-NMR spectrum of peptide563-PEtOx$_{3600}$-*b*-PLA$_{14400}$.

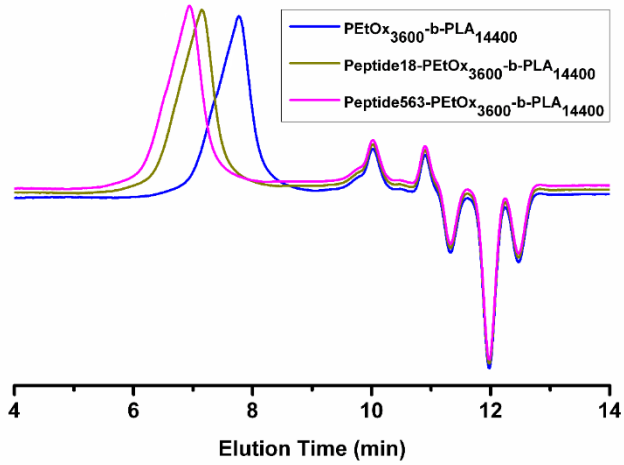


Figure 8. The GPC chromatogram of peptide conjugated PEtOx$_{3600}$-*b*-PLA$_{14400}$ amphiphilic block copolymers.

4. Conclusion

As outlined in the introduction part, previous reports documented the synthesis poly(2-ethyl-2-oxazoline)-*b*-poly(L-lactide) in a modular way through the use of conventional Click chemistry (**Scheme 1A**) [8]. When viewed from the targeted drug delivery system, this approach is rather

inconvenient in that it does not effectuate a functional group on hydrophilic block (i.g. PEtOx) to conjugate tumour-homing agents. Contrary to this approach, our methodology desirably generates a functional group on the hydrophilic block to which the tumour-homing agents could be tethered through thiol-ene reaction (**Scheme 1B**). In overall, the methodology we report hereby is conformed with the synthesis of the building blocks, such as **1a** and **1b** to tailor targeted drug delivery system. The fabrication of polymeric particles with these novel materials is currently in progressin our laboratories.

5. Acknowledgements

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aqueous solutions, and encapsulation of the hydrophobic drug dipyridamole. Biomacromolecules 2010; 11: 430-438. doi: 10.1021/bm901151g

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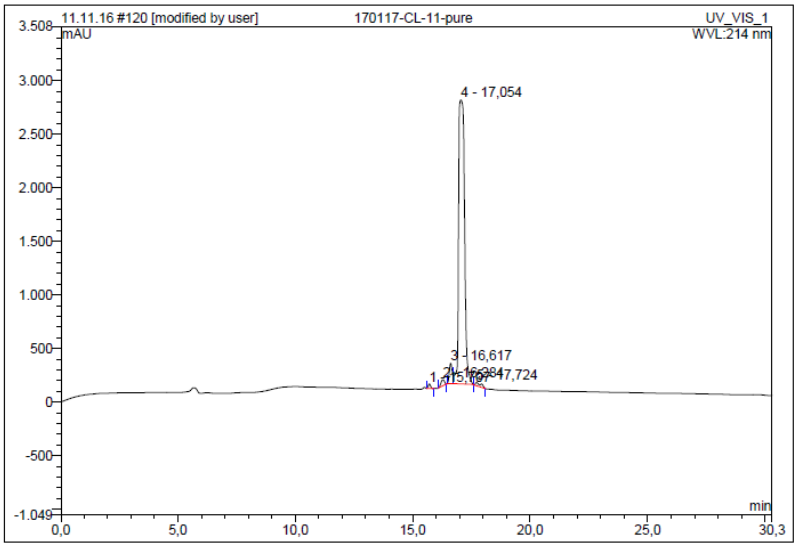
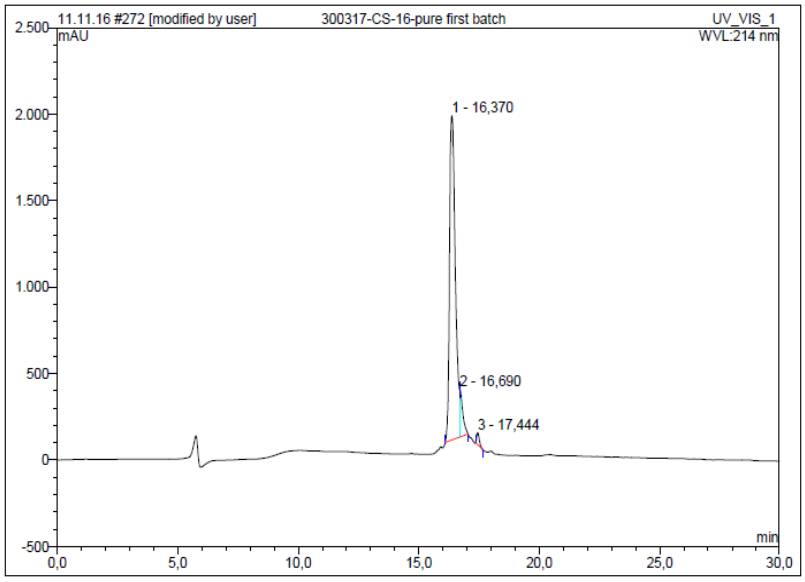
Supporting Information



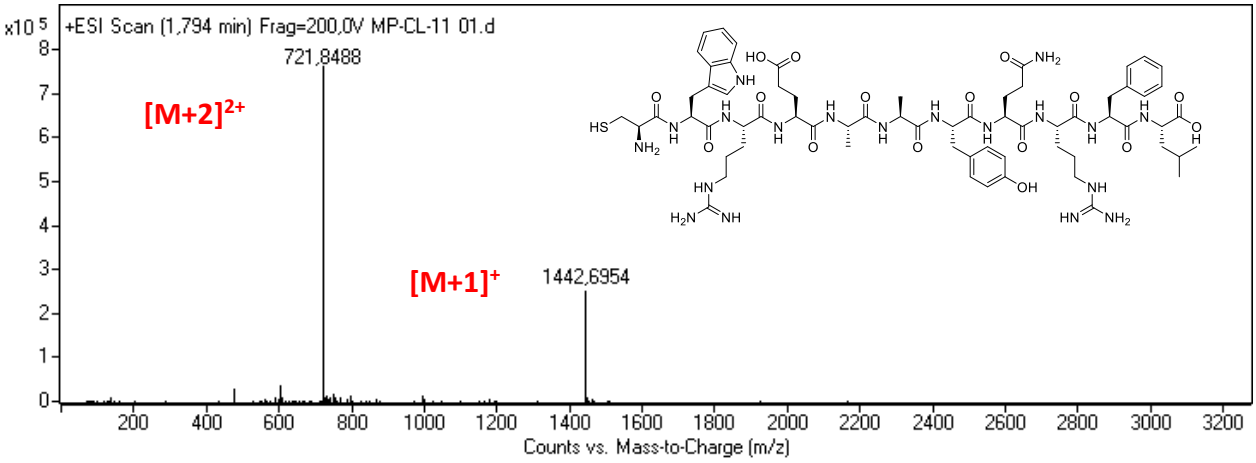
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	15,71	n.a.	41,610	4,801	0,58	n.a.	BMB*
2	16,28	n.a.	54,881	9,257	1,12	n.a.	BMB*
3	16,62	n.a.	189,312	27,020	3,27	n.a.	BM *
4	17,05	n.a.	2649,939	776,605	93,93	n.a.	MB*
5	17,72	n.a.	30,205	9,130	1,10	n.a.	BMB*
Total:			2965,947	826,814	100,00	0,000	

Figure S1. The RP-HPLC chromatogram of peptide-18.



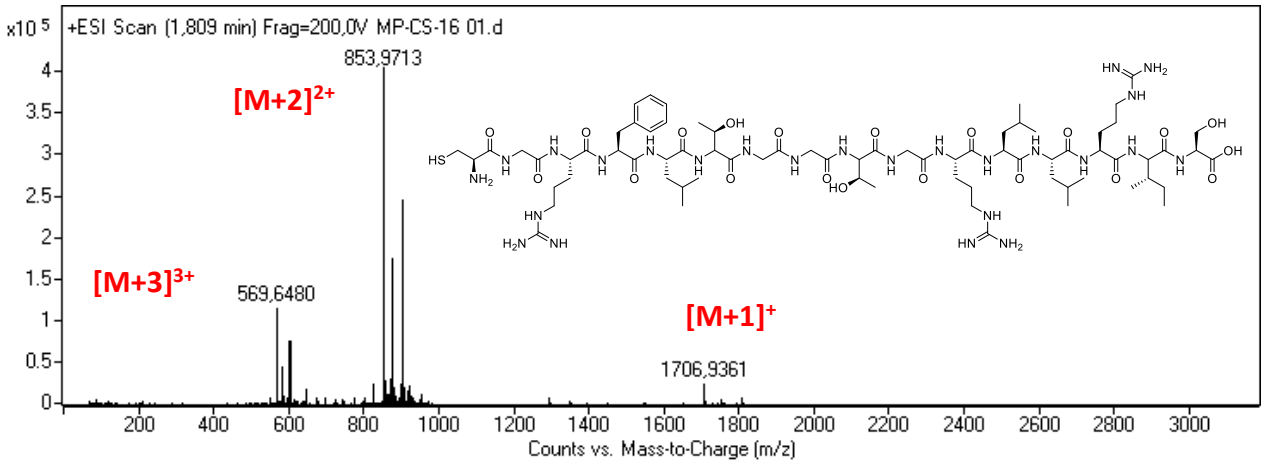
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	16,37	n.a.	1875,011	518,137	91,25	n.a.	BM *
2	16,69	n.a.	275,120	41,007	7,22	n.a.	MB*
3	17,44	n.a.	69,379	8,708	1,53	n.a.	BMB*
Total:			2219,510	567,852	100,00	0,000	

Figure S2. The RP-HPLC chromatogram of peptide-563.



Entry	Sequence	Molecular Formula	[M+1]+ calculated	[M+2]+ calculated
Pep-18	H-CWREAAYQRFL-OH	$C_{66}H_{95}N_{19}O_{16}S$	1442.6925	722.3463

Figure S3. The mass spectrum of peptide-18.



Entry	Sequence	Molecular Formula	$[M+1]^+$ calculated	$[M+2]^+$ calculated
Pep-563	H-CGRFLTGGTGRLLRIS-OH	$C_{73}H_{127}N_{25}O_{20}S$	1706.9410	854.4705

Figure S4. The mass spectrum of peptide-563.

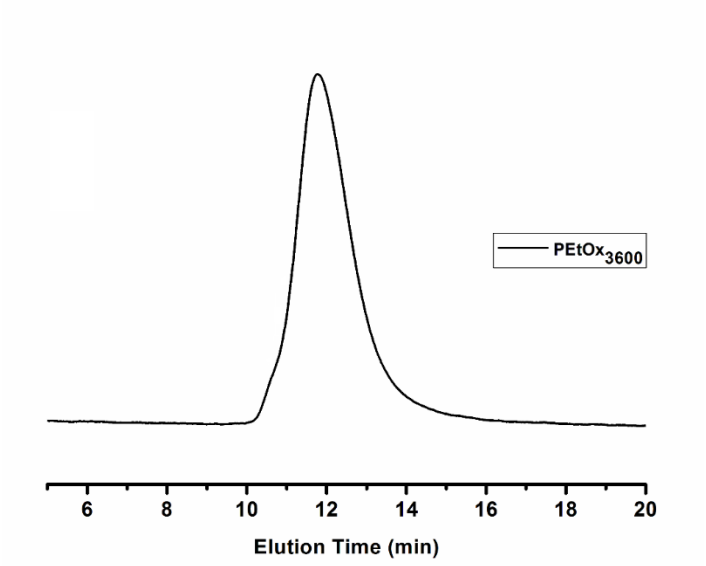


Figure S5. The GPC chromatogram of $PEtOx_{3600}$.