International Journal of Pharmaceutics 466 (2014) 58–67

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

An *in vivo* evaluation of amphiphilic, biodegradable peptide copolymers as siRNA delivery agents

Stephanie E. Barrett [a,*], Marc T. Abrams [b], Rob Burke [c], Brian A. Carr [c], Louis S. Crocker [d], Robert M. Garbaccio [a], Bonnie J. Howell [b], Eric A. Kemp [d], Robert A. Kowtoniuk [a], Andrew H. Latham [d], Karen R. Leander [b], Anthony M. Leone [d], Mihir Patel [d], Sergey Pechenov [d], Nicole T. Pudvah [c], Sean Riley [d], Laura Sepp-Lorenzino [b], Eileen S. Walsh [b], J. Michael Williams [a], Steven L. Colletti [a]

[a] Department of RNA Medicinal Chemistry, Merck Research Laboratories, Merck & Co., Inc., West Point, PA 19486, USA
[b] Department of RNA Biology, Merck Research Laboratories, Merck & Co., Inc., West Point, PA 19486, USA
[c] Department of Pharmacokinetics & Drug Metabolism RNA, Merck Research Laboratories, Merck & Co., Inc., West Point, PA 19486, USA
[d] Department of RNAi Analytical Sciences, Merck Research Laboratories, Merck & Co., Inc., West Point, PA 19486, USA

ARTICLE INFO

Article history:
Received 8 December 2013
Received in revised form 28 February 2014
Accepted 2 March 2014
Available online 05 March 2014

Keywords:
siRNA
RNA delivery
Polymer conjugates
Poly(peptides)

ABSTRACT

A series of amphiphilic, biodegradable polypeptide copolymers were prepared for the delivery of siRNA (short interfering ribonucleic acid). The molecular weight (or polymer chain length) of the linear polymer was controlled by reaction stoichiometry for the 11.5, 17.2, and 24.6 kDa polypeptides, and the highest molecular weight polypeptide was prepared using a sequential addition method to obtain a polypeptide having a molecular weight of 38.6 kDa. These polymers were used to prepare polymer conjugate systems designed to target and deliver an apolipoprotein B (ApoB) siRNA to hepatocyte cells and to help delineate the effect of polymer molecular weight or polymer chain length on siRNA delivery *in vivo*. A clear trend in increasing potency was found with increasing molecular weight of the polymers examined (at a constant polymer:siRNA (w/w) ratio), with minimal toxicity found. Furthermore, the biodegradability of these polymer conjugates was examined and demonstrates the potential of these systems as siRNA delivery vectors.

1. Introduction

Since the discovery of RNA interference (RNAi) by Fire and Mellow in 1998 (Fire et al., 1998), both academic and industrial scientists worldwide have been working towards the realization of siRNA as a therapeutic option for patients. The key challenge that all scientists face is delivery of the siRNA. Successful systemic delivery of siRNA into cells depends on the ability of the delivery vehicle to protect siRNA from degradation while in circulation, deliver the genetic material to the target cell of interest, and assist the siRNA with endosomal escape (Fig. 1) (Whitehead et al., 2009; Stanton and Colletti, 2010; Zimmermann et al., 2006; Convertine et al., 2009; Itaka et al., 2004; Wang et al., 2008, 2007). Polymer conjugates have proven to be an effective targeted delivery strategy, offer small sizes for improved tissue penetration (diameters <50 nm), and provide a high degree of chemical control over the structure of the conjugate (Convertine et al., 2009; Rozema et al., 2007; Matsumoto et al., 2008; Gaspar and Duncan, 2009). Various polymer-based carriers have been developed for this purpose, but most suffer from toxicity derived from the carrier components, limiting the practical application of such systems (Gaspar and Duncan, 2009; Fischer et al., 2003; Morille et al., 2008; Kichler, 2004; Kircheis et al., 2001).

A recent report describing a dynamic polyconjugate delivery system for siRNA that demonstrated *in vivo* messenger RNA (mRNA) knockdown in mouse liver was described by Rozema *et al.* (Rozema et al., 2007). This design employed non-degradable cationic amphiphilic endosomolytic polymers as their backbones with a reversible disulfide bond to siRNA and pH-sensitive masking groups to mitigate polycation-associated toxicity. The disulfide link to the siRNA is thought to be stable in circulation, but to release siRNA in cells where glutathione concentrations are increased. The pH-sensitive masking groups (carboxydimethyl maleic anhydride or CDM) possessed a targeting ligand (*N*-acetylgalactosamine, GalNAc) to promote uptake into parenchymal liver cells using the asialoglycoprotein receptor (ASGPr) or a stealth agent (poly

* Corresponding author. Tel.: +1 215 652 7348; fax: +1 215 652 7310.
E-mail address: stephanie_barrett@merck.com (S. E. Barrett).

http://dx.doi.org/10.1016/j.ijpharm.2014.03.011
0378-5173/

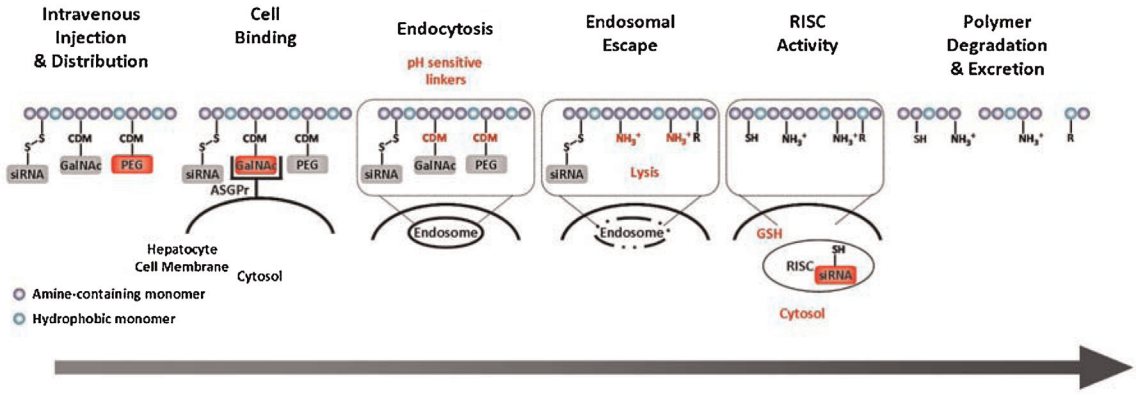


Fig. 1. The process of *in vivo* siRNA delivery using targeted siRNA-polymer conjugates. Components highlighted in red are thought to be the most significant constituent for that particular step.

(ethylene glycol), or PEG) to avoid unfavorable interactions in circulation. Successful delivery of the siRNA cargo depends on delivery to the target cell of interest, receptor mediated endocytosis and then endosomal acidification that results in demasking and endosomal escape. Following disulfide cleavage, the siRNA in the cytosol is able to load into the RNA-induced silencing complex (RISC) and engage in RNA interference. In this delivery system, the polymer plays a crucial role as the vehicle for the drug (i.e., siRNA), the targeting ligand (GalNAc), and the stealth agent (PEG) and must be synthesized from biodegradable components that can be excreted from the body once the delivery of siRNA to the target cells is complete.

A potential limitation of this design is the non-degradable nature of the poly(vinyl ether) (PBAVE) endolytic polymers that have the potential to induce acute and chronic toxicity once demasked. In contrast to these PBAVE-based systems, the poly(peptides) employed in this study bear several advantages including the potential for biodegradability and excretion, as well as control over monomer incorporation, polymer architecture and polydispersity (Sun et al., 2011). By virtue of a living polymerization, the synthesis of poly(peptides) allows for tremendous diversity to be built into the side chain functionality of the polymer (Deming, 2000; Habraken et al., 2011; Vayaboury et al., 2004), along with improved maneuverability in chemical space available for improving potency and reducing toxicity. The poly(peptide) polymers presented herein were synthesized from *N*-carboxyanhydrides via ring-opening polymerization using high-vacuum techniques with a primary amine initiator (Hadjichristidis et al., 2009; Pickel et al., 2009; Aliferis et al., 2004; Habraken et al., 2011).

This study assesses the potential of poly(peptides) to serve as biodegradable delivery vehicles for siRNA. In order to obtain efficient endosomal escape, it was thought that the appropriate amphiphilicity or balance of hydrophilic to hydrophobic monomers must be met and the two monomer components chosen for this initial study were L-ornithine and L-phenylalanine. Both monomers represent natural α-amino acids, with the potential of being degraded enzymatically *in vivo*. Additionally, L-ornithine has the potential to undergo spontaneous lactamization once *in vivo* as an alternative degradation pathway (Weber and Miller, 1981). It was hypothesized that this lactamization process could aid in the overall biodegradability of polymer, which is likely to occur after the polyconjugate had undergone endocytosis, demasked and affected endosomal escape.

Given the potential for endopeptidase and exopeptidase degradation, we suspected that polymer molecular weight or polymer chain length may be an important component of the structure activity relationship for polymer based siRNA delivery to balance the need for liver targeting and siRNA delivery with the inevitable polymer degradation that will occur once the system is *in vivo*. In this study, siRNA polymer conjugates were prepared using biodegradable polymers at various molecular weights or polymer chain lengths to determine the effect of molecular weight on siRNA delivery. These polymers were conjugated to siRNA, and masked with CDM-based targeting ligands and PEG that are labile and triggered by the chemical environment of the endosome and cytosol. Once released in the acidified endosomal component, the endosomolytic polymer aids in the endosomal escape of the siRNA only then to be degraded *in vivo*.

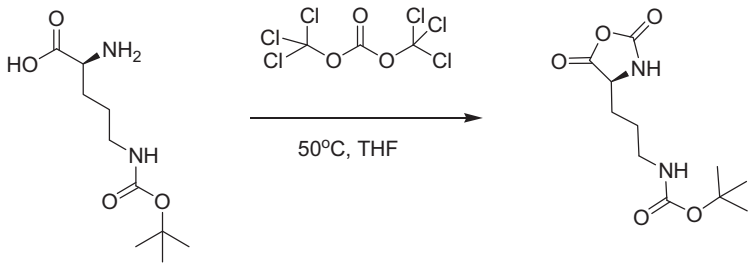
2. Materials and methods

2.1. Materials

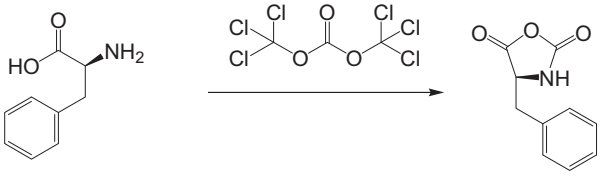
N^{d}-Boc-L-ornithine was obtained from Chem-Impex International (product# 05367). L-phenylalanine, triphosgene, *n*-butylamine, tetrahydrofuran (THF), cyclopentylmethyl ether (CPME), hexanes, anhydrous *N*,*N*-dimethylacetamide (DMA), anhydrous dimethylsulfoxide (DMSO), dichloromethane (DCM), trifluoroacetic acid (TFA), tris(hydroxymethyl) aminomethane (TRIS), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, (HEPES), glucose, sucrose, sodium chloride (NaCl), *N*,*N*-dimethylformamide (DMF), lithium chloride (LiCl), poly(styrene) gel permeation chromatography (GPC) standards, acetonitrile (ACN), tyloxapol (Triton WR1339), and *Streptomyces griseus* were purchased from Sigma–Aldrich. 4-Succinimidyloxycarbonyl-alpha-methyl-alpha (2-pyridyldithio) toluene (SMPT), the BCA (bicinchoninic acid) assay kit, and DyLight 650 NHS ester was obtained from Thermo Scientific. Female Sprague-Dawley rats were purchased from Charles River. Carboxydimethylmaleic anhydride-*N*-acetylgalactosamine (CDM-GalNAc) and carboxydimethylmaleic anhydride poly(ethylene glycol) (CDM-PEG) were prepared according to literature procedures (Rozema et al., 2007).

2.2. Preparation of Boc-L-ornithine N-carboxyanhydride (NCA) (Scheme 1)

A 2 L round bottom was dried in an oven prior to use (oven temp = 120 °C). The glassware was cooled under an inert nitrogen atmosphere. Boc-L-ornithine (35 g, 151 mmol) was added to the flask, along with anhydrous THF (1.2 L, 0.13 M). To the slurry was added a solution of triphosgene (16.6 g, 55.8 mmol, 0.37 equivalents) in anhydrous THF (240 mL, 0.6 M). The reaction was heated at 50–55 °C for 1 h then cooled to ambient temperature. The remaining solid was removed by filtration washing with 100 mL of THF. The filtrate was concentrated by vacuum distillation to 350 mL



Scheme 1. Synthesis of boc-L-ornithine *N*-carboxyanhydride.



Scheme 2. Synthesis of L-phenylalanine *N*-carboxyanhydride.

and the solvent was switched to cyclopentylmethyl ether (CPME, 350 mL). The resulting slurry was cooled to ambient temperature and stirred under nitrogen overnight. The solid was isolated by filtration washing with 70 mL of CPME and vacuum dried to give a white crystalline product (35 g, 92%). Solid was collected and stored at −20 °C in a sealed bottle. GC/MS was used to confirm the product. ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.08 (s, 1 H); 6.86 (s, 1 H); 4.44 (t, J = 6.15 Hz, 1 H); 2.92 (t, J = 6.41 Hz, 2 H); 1.75 – 1.67 (m, 1 H); 1.65 – 1.57 (m, 1 H); 1.51 – 1.30 (m, 2 H); 1.38 (s, 9 H) .

2.3. Preparation of L-phenylalanine N-carboxyanhydride (NCA) (Scheme 2)

A 1 L round bottom was dried in an oven prior to use (oven temp = 120 °C). The glassware was cooled under an inert nitrogen atmosphere. Phenylalanine (50.0 g, 303 mmol) was added to the flask. Anhydrous THF (600 mL, 0.5 M) was charged to give a suspension of white solid. The mixture was heated to 50 °C and triphosgene (35.9 g, 121 mmol, 0.4 equivalents) was added as a solid. The suspension was stirred until the reaction was clear (∼30 min). The reaction mixture was cooled to ambient temperature then concentrated to an oil. The oil was then slowly poured into 3 L of hexanes with rapid stirring to yield a white precipitate. The resulting suspension was capped using aluminum foil and placed in the freezer for 3 h. The white precipitate was then filtered via vacuum filtration while maintaining an inert environment. The white solid was rinsed with hexanes (3 × 20 mL) to give the product. The white solid was collected and dried overnight under vacuum. GC/MS was used to confirm the product (54.0 g, 94% yield). ^{1}H NMR (500 MHz, $CHCl_3$-*d*): δ 7.37 – 7.30 (3 H, m); 7.18 (2 H, d, J = 7.21 Hz); 6.23 (1 H, s); 4.53 (1 H, dd, J = 8.18, 4.21 Hz); 3.27 (1 H, dd, J = 14.14, 4.20 Hz); 3.00 (1 H, dd, J = 14.13, 8.17 Hz) .

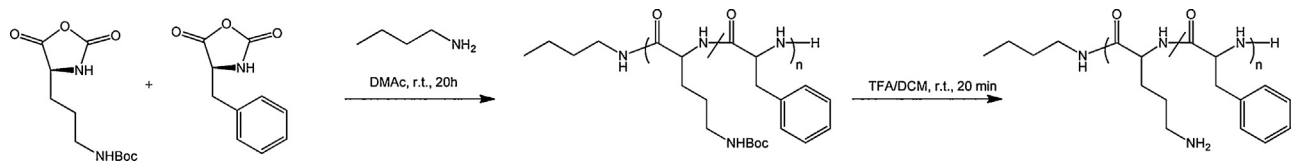
2.4. General polymer synthesis

In a typical polymerization, L-Boc-ornithine-*N*-carboxyanhydride (1 g, 3.87 mmol) and phenylalanine-*N*-carboxyanhydride (0.170 g, 0.891 mmol, 0.23 equivalents) were placed in an oven-dried round-bottom flask and the flask was purged with an atmosphere of nitrogen. 10 mL of anhydrous DMA (water content = 68 ppm) was added and the solution was stirred until it became clear. *N*-butylamine (11 μL, 0.116 mmol, 0.03 equivalents) was added to the roundbottom as a solution and the entire flask was put under vacuum (<10^{-6} mmHg). The solution bubbled, suggesting the release of CO_2. The solution continued to stir at room temperature overnight. The next day, the solution was clear. The polymer solution was precipitated in water (20× reaction volume) and then filtered. The collected precipitate was frozen and placed on the lyophilizer for 48 h to dry the product. Table 1 shows the exact amounts added of each component for each polymerization. The last line in the table shows the amount added for the polymer having the highest molecular weight, where a second aliquot of monomers (denoted as 4′) was added the following morning along with an additional 5 mL of DMA and allowed to polymerize for an additional 8 h at room temperature, under vacuum. This sequential addition was required to achieve the highest molecular weight and was prepared according to literature procedures (Kowtoniuk et al., 2014).

The protected polymer was added to a round bottom and dissolved in dichloromethane (35 mg/mL polymer). The solid dissolved readily to give a hazy solution. The solution was stirred at room temperature under nitrogen. Trifluoroacetic acid was added to the solution (1:1 dichloromethane:trifluoroacetic acid by volume). The solution became clear immediately and was allowed to react for 20 min. The deprotected polymer was obtained as a trifluoroacetic acid salt after the solvent and volatile byproducts were removed by vacuum (Scheme 3).

Table 1
Material requirements for polymerization.

Sample	L-ornithine NCA (g, mmol)	L-phenylalanine NCA (g, mmol)	DMA (mL)	*n*-butyl amine (μL, mmol)
1	1, 3.87	0.170, 0.891	10	11, 0.116
2	1, 3.87	0.170, 0.891	10	3.83, 0.039
3	1, 3.87	0.170, 0.891	10	1.91, 0.019
4	1, 3.87	0.170, 0.891	10	1.91, 0.019
4′	0.770, 2.98	0.131, 0.686	5	0, 0



Scheme 3. Synthesis of linear amphiphilic statistical copolymers by living ring-opening polymerization.

2.5. GPC analysis

Molecular weight and molecular weight distributions were estimated using a gel-permeation chromatography (GPC) (Waters Alliance 2695 Separations Module) system equipped with a TOSOH TSKgel Alpha 3000 column and a Waters 2414 refractive index detector. The columns were eluted with dimethylformamide (DMF) containing lithium chloride (10 mM) (0.5 mL/min) at 40 °C. The molecular weights and molecular weight distributions of poly (amide) polymers were compared to poly(styrene) standards (Sigma–Aldrich).

2.6. General polyconjugate synthesis

Step 1: Activation of polymer. Polymer (122 mg, 41 wt% purity with the major component being TFA) was dissolved in 1.67 mL of anhydrous DMSO. The solution was mixed until the polymer was completely dissolved and 150 μL of a solution of 4-succinimidyloxycarbonyl-alpha-methyl-alpha(2-pyridyldithio) toluene (SMPT) in DMSO (1 mg/100 μL) was added (corresponding to 1.5 wt% with respect to the polymer weight).

Step 2: Activation of oligonucleotide. Amine terminated Sci10 ApoB oligonucleotide (1 g, 0.0714 mmol) was dissolved in 0.1 M sodium bicarbonate buffer (20 ml, 50 mg/mL) in a vial with magnetic stir bar and cooled to 0–5 °C in an ice water bath. In a separate vial, *N*-succinimidyl S-acetylthioacetate (SATA) (83 mg, 0.357 mmol, 5 equivalents) was dissolved in 0.78 mL of DMSO. The SATA solution was added over 1 min and the clear, colorless reaction mixture was stirred at 0–5 °C. After 2 h, the reaction mixture was sampled and analyzed by UPLC or HPLC (Dionex DNApac) for completion of the reaction. Additional SATA can be added to effect complete conversion of the oligonucleotide (<5% remaining unreacted). The reaction mixture was purified by tangential flow filtration (TFF) using water (~2 L). The retentate was lyophilized to give a white solid. The recovery was ~95% and the purity was greater than 70% by UPLC.

Step 3: Polymer-oligonucleotide conjugation. The entire sample of activated polymer from Step 1 was diluted with 19.7 mL of 100 mM TRIS, 5% glucose buffer at pH 9 resulting in a final polymer concentration of ~2.5 mg/mL. 10 mg of oligonucleotide was added to the activated polymer solution and allowed to react at room temperature for one hour until the final masking step. *In situ*, the primary amine on the polymer is assumed to deprotect the SATA modified siRNA to produce the free thiol siRNA, which can then react with the SMPT-modified polymer.

Step 4: Masking of the polymer conjugate. In a separate vial, 103 mg (0.218 mmol, 50 equivalents) of carboxydimethylmaleic anhydride-N-acetylgalactosamine (CDM-GalNAc) and 444 mg (0.653 mmol, 150 equivalents) of carboxydimethylmaleic anhydride poly(ethylene glycol (CDM-PEG) were weighed out. The siRNA-polymer conjugate solution was then transferred into the vial containing CDM-GalNAc and CDM-PEG and the resulting solution was stirred at room temperature for 10 min. The final pH of the polyconjugate solution was 8.3–8.5.

Step 5: Purification of the polymer conjugate. Tangential flow filtration (TFF) was used to purify polymer conjugate formulations of unincorporated components and to exchange buffer to a pharmaceutically acceptable formulation vehicle using a TFF system. The TFF filter material was made of regenerated cellulose. The selection of molecular weight cutoff for these membranes was chosen with efficiency of purification and retention of polymer conjugate in mind. The processing parameters included feed pressure, retentate pressure, crossflow rate and filtrate flux and were set to allow reproducibility from batch to batch and linear scaling of the process. Using the difiltration mode of TFF, the reaction impurities were filtered out into the permeate and the buffer for the retained polymer conjugate was exchanged. After TFF, the final product was concentrated to 0.4–2.0 mg/mL of siRNA and sterile filtered using a 0.2 μm PES syringe filter and stored at −20 °C until use (Scheme 4).

2.7. siRNA conjugation efficiency

Free RNA duplex as well as free RNA duplex-dimer was determined by aqueous SEC using a GE Healthsciences Superdex 75HR 10/300 column. The mobile phase was composed of 100 mM TRIS with 2 M NaCl, pH 8.4. Total RNA (both free and bound) was determined by using inductively coupled plasma (ICP) spectroscopy. Since the RNA is the only phosphorus containing species in the formulations, determining the total phosphorus content can be used to directly determine the total RNA concentration. Once the free RNA (duplex and duplex-dimer) and total RNA is determined, the amount of RNA conjugated to the polymer can be calculated (i.e., conjugation efficiency, see Supporting Information Figures S1 and S2).

2.8. Masking efficiency

Total concentrations of CDM-GalNAc and CDM-PEG were determined using reverse-phase HPLC with mobile phases of 0.1% TFA in water and 0.1% TFA in acetonitrile with UV detection. Rapid demasking of the polymer after injection onto the column allowed quantitation of CDMs with the polymer removed using a C18 guard column to prevent chromatographic interference. Free (i.e., unbound) CDM-GalNAc and CDM-PEG was analyzed by first filtering through a 10 kDa centrifuge filter followed by analysis using the same reverse-phase HPLC method. Masking efficiency was calculated by first calculating the bound RNA, CDM-GalNAc and CDM-PEG. The polymer molecular weight in combination with the total amines available for conjugation was then used with the bound ligands to calculate masking efficiency (see Supporting Information Fig. S3).

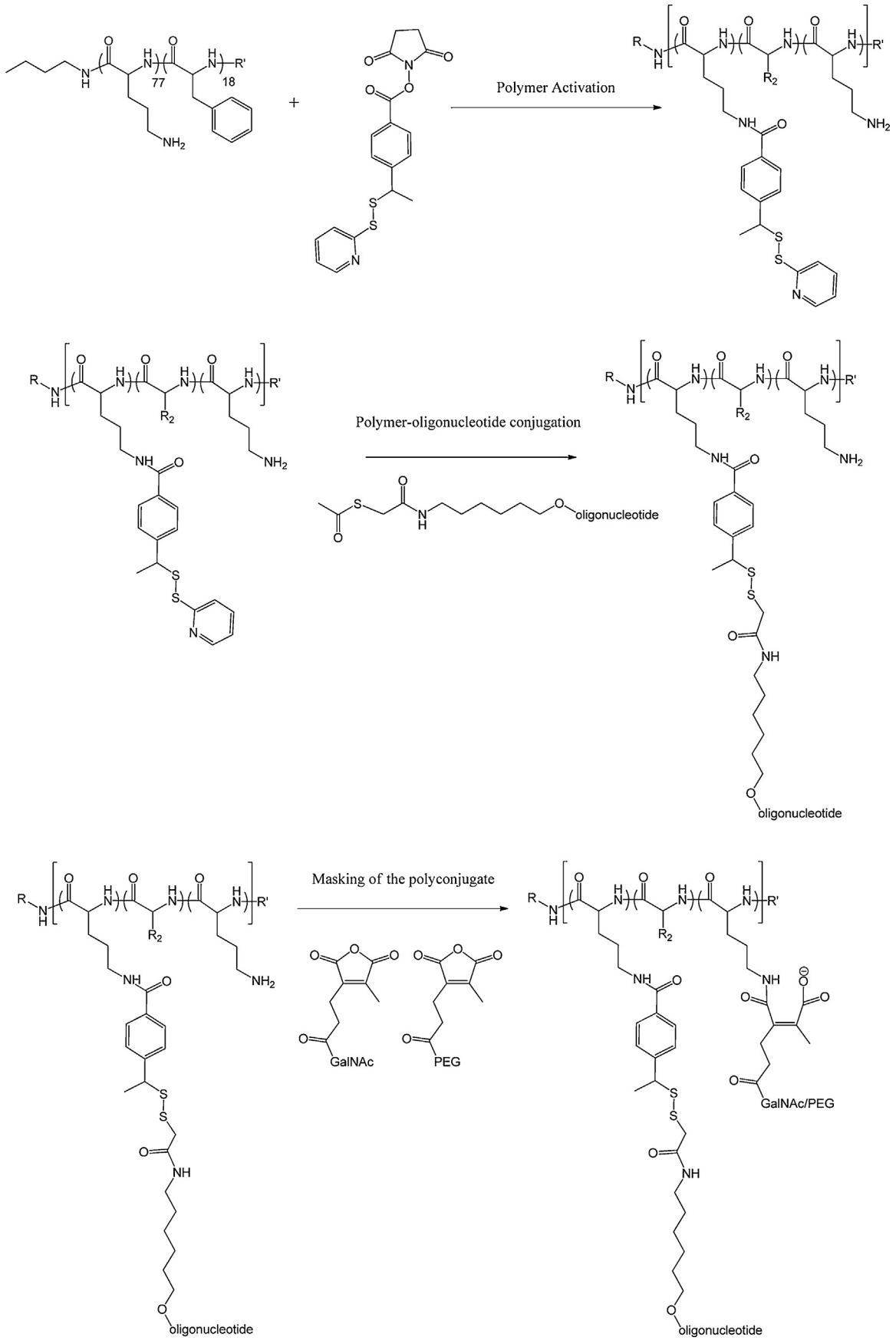
2.9. Polymer assay

Polymer purity was determined by using a LECO TruSpec N nitrogen combustion analyzer. Approximately 50 mg of polymer was combusted in an atmospher using the LECO TruSpec N software (v1.26). Quantitation of nitrogen was achieved by using urea as an external standard. Instrument parameters are summarized in the supporting information section (Table S1). Polymer purity (free base) was calculated as the ratio between the measured nitrogen content and the stoichiometric amount of nitrogen.

With polymer purity established, an accurate HPLC standard was prepared by dissolving the polymer in water. Masked polymer-conjugated siRNA test articles were treated with dithiothreitol (DTT), followed by a two-step dilution with 0.1 N NaOH and 4 M NaCl dissolved in 10 mM HCl. The final method concentration for the polymer assay was 0.04 mg/mL (free base). Samples and standards were analyzed using an Agilent 1100 with an online NQAD 500 (Quant Technologies) universal detector. All instrument control and data analyses were performed using the Thermo Atlas Chromatography Data System (v8.2). Quantitation of nitrogen was achieved by using an external standard. Method conditions are summarized in the Supporting Information section (Table S2).

2.10 In vivo *experiments*

Sci10 ApoB siRNA was utilized in the experiments.
Sci10 ApoB siRNA



Scheme 4. Synthesis of siRNA-polymer conjugates.

5′-amino-iB-CUUU*AA*C*A*AUUCCU*GAAA*U**T**s**T**-iB-3′ (SEQ ID NO.:1)

3′-Us*U**GAAA**U**UG*UU*AAGGA*CUsUsUsA-5′ (SEQ ID NO.:2)

Bases: A – Adenine; U – Uracil; G – Guanine; C – Cytosine; T – Thymine. Modifications to the sugars: iB – Inverted deoxy abasic; *AGU* – 2′ Fluoro; **T** – 2′ Deoxy; CU – 2′ OCH_3; s – phophorothioate linkage between nucleotides.

Doses were administered to rats based on the weight of siRNA. Five days post intravenous tail vein injection, the Sprague-Dawley rats were sacrificed and liver tissue samples were immediately preserved in RNALater (Ambion). Preserved liver tissue was homogenized and total RNA isolated using a Qiagen bead mill and the Qiagen miRNA-easy RNA isolation kit following the manufacturer's instructions. Liver ApoB mRNA levels were determined by quantitative reverse transcription polymerase chain reaction (RT-PCR). Message was amplified from purified RNA utilizing primers against the mouse ApoB mRNA (Applied Biosystems). The PCR reaction was run on an ABI 7500 instrument with a 96-well Fast Block. The ApoB mRNA level was normalized to the housekeeping genes peptidyl isomerase B (PPIB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PPIB and GAPDH mRNA levels were determined by RT-PCR using a commercial probe set (Applied Biosytems). Results are expressed as a ratio of ApoB mRNA/ PPIB / GAPDH mRNA. All mRNA data is expressed relative to the vehicle control.

Alanine aminotransferase (ALT) was measured using the ADVIA Chemistry Systems Alanine Aminotransferase (ALT) method, 03815151, Rev. A (Berte et al., 2006). All animal studies were conducted according to approved Institutional Animal Care and Use Committee (IACUC) protocols.

2.11. Preparation of fluorophore-labeled polymers

A statistical copolymer of L-ornithine and L-phenylalanine was prepared for these labeling experiments. Here, poly(ethylene glycol)-amine (2 kDa) was used to initiate the polymerization, and the polymerization and characterization proceeded according to the procedure described above. The final polymer showed a number-average molecular weight of 11,400 g/mol and a PDI (polydispersity index) of 1.12 (DP = degree of polymerization, $DP_{ORN} = 74$, $DP_{PHE} = 20$).

A solution of polymer (200 mg, 0.0018 mmol) was prepared in anhydrous DMSO (5 mL, 3.51 mM). Separately, a solution of DyLight 650 (Thermo Pierce) (1.29 mg, 1.28 μmol, 0.07 equivalents) was prepared in anhydrous DMSO (0.2 mL, 6.40 mM). The entire solution of fluorophore was then added to the solution of polymer and allowed to react at room temperature overnight. The fluorophore-labeled conjugate was then dialyzed using a 10,000 g/mol molecular weight cut-off centrifuge dialysis membrane (centrifuged at 3800 × *g* = 4600 rpm) against 100 mM TRIS, pH 9, with 1.5 N NaCl (four times). The labeled polymer was then dialyzed four additional times using distilled water. Using water, the polymer was transferred and filtered through a 0.22 um filter into a pre-weighed scintillation vial, and lyophilized to dryness yielding a blue powder (85 mg, 43%).

2.12. Lysosomal lysate generation

Tritosomes, which are highly representative of lysosomes, were isolated using a modified protocol based on published methods of Bagshaw *et al.* (Bagshaw et al., 2003) Briefly, female Sprague-Dawley rats (Charles River) were dosed at 450 mg/kg body weight with tyloxapol (Triton WR1339; Sigma #T0307) prepared in normal saline via intraperitoneal injection. Five days post-dose, animals were sacrificed using CO_2 and livers were harvested, flushed with saline via hepatic portal vein, rinsed in saline, and minced into 8 g pieces. Livers were homogenized in 0.25 M sucrose with 20 mM HEPES (pH 7.4) at a 1:3 (w/v) ratio using a glass/Teflon Potter-Elvehjem tissue grinder. The liver homogenates were centrifuged at 1000 × *g* for 10 min using an Avanti J25I rotor (Beckman) and the post-nuclear supernatant was collected. This supernatant was then centrifuged at 34,000 × *g* for 10 min using the same rotor, the fat layer and supernatant were discarded, and the organelle pellet was resuspended in 45% sucrose. Ultracentrifuge tubes (Beckman #344058) were prepared with a discontinuous gradient of 14.3% sucrose and 34.5% sucrose, the organelles in 45% sucrose were underlayed beneath the gradient, and then the tubes were centrifuged at 77,000 × *g* for 2 h at 4 °C in a SW-28 rotor (Beckman). Tritosomes were collected from the interface between the 14.3% and 34.5% sucrose layers, diluted 1:2.5 in 0.25 M sucrose with 20 mM HEPES, and centrifuged at 28,000 × *g* for 30 min at 4 °C. The pellet containing the tritosomes was then resuspended in 0.25 M sucrose with 20 mM HEPES, the tritosomes were lysed using a sonic dismembrator (Fisher) followed by one freeze/thaw cycle, and the protein concentration was determined using a BCA assay kit (Pierce #23227) according to the manufacturer's instructions. To ensure that the tritosome preparations were representative of lysosomes, the activity of citrate synthase (a mitochondrial enzyme) and acid phosphatase (a lysosomal enzyme) were measured by enzyme assay kits (Sigma #CS0720 and #CS0740, respectively) according to the manufacturer's instructions.

During normal differential centrifugation to isolate specific organelles, it can often be very difficult to separate lysosomes from other organelles (especially mitochondria) due to the organelles having very similar densities. The methods used in this work are based upon the uptake and trafficking of Tyloxapol (Triton WR1339) to the lysosomes of hepatocytes, which alter the density of the lysosomes and enable a very simple isolation of liver lysosomes using a discontinuous sucrose gradient. Testing the tritosome preparations for enzyme activity, it was determined that the tritosomes had no measurable citrate synthase activity and ~1 unit/mg protein of acid phosphatase activity (average of multiple batches), thus confirming that the tritosome preparations are highly representative of lysosomes and not of mitochondria.

2.13. Gel electrophoresis-based polymer degradation assay

A cocktail of bacterial proteases from *Streptomyces griseus* (Sigma #P5147, also called Pronase E) was used as a degradation matrix in addition to the lysosomes prepared above. Fluorescently labeled free polymer or polyconjugate was spiked into these matrices at a final polymer concentration of 0.02 mg/mL, with the matrices diluted to a protein concentration of 1 mg/mL and buffered to pH 6.0 with 20 mM TRIS. One parent reaction vial was incubated at 37 °C and a 10 uL aliquot was removed at each desired time point (0, 2, 4, 6, and 24 h) and flash frozen on dry ice. After completion of the incubations, the samples were thawed, each aliquot was mixed 1:1 (v/v) with dye-free gel loading buffer (450 mM TRIS HCl, 12% Glycerol, and 4% sodium dodecyl sulfate (SDS) at pH 8.45), and these mixtures were heated at 85 °C for 2 min. After cooling, 10 uL of each sample was loaded into the wells of a Novex 16% Tricine gel (Invitrogen #EC66955BOX) and run at 125 V for 60 min using a 1X Novex Tricine SDS running buffer (Invitrogen # LC1675). After running, the gel was quickly rinsed with distilled H_2O and then imaged with a FluorChem Q MultiImage III (Alpha Innotech) with the excitation/emission channel set to Cy5 and using the autoexposure settings within the AlphaView Q software (Alpha Innotech).

Table 2
Characterization of the (L-ornithine:L-phenylalanine) statistical copolymers.

Sample	*Mn* of the protected polymer (g/mol)	PDI of the protected polymer (from DMF GPC)	Projected *Mn* after deprotection (g/mol)	Absolute *MW* from aqueous GPC (g/mol)	DP_{ORN}	DP_{PHE}	L-orn:L-phe (NMR ratio)	Wt% purity by nitrogen analysis
1	19,200	1.11	11,500	7400	77	18	4.17	41
2	28,300	1.11	17,200	11,700	112	30	3.70	40
3	41,100	1.19	24,600	22,400	165	40	4.16	nd
4‘	64,300	1.09	38,600	32,300	258	63	4.12	43

Mn = number – average molar mass; *MW* = absolute molecular weight; PDI = polydispersity index; GPC = Gel permeation chromatography; DP = degree of polymerization as determined by ^{1}H NMR (see Supporting Information for calculations); nd = not determined).

3. Results and discussion

3.1. Polymer design and synthesis

To investigate the effect of molecular weight or polymer chain length of linear amphiphilic copolymer structures on their ability to knockdown mRNA, we prepared a series of amphiphilic poly(L-ornithine:L-phenylalanine) statistical copolymers from the ring-opening polymerization of *N*-carboxyanhydrides (NCA). This polymerization method has been previously used to prepare living polymers under vacuum conditions (Habraken et al., 2011; Hadjichristidis et al., 2009). In this study, the polymers were prepared from the addition of a boc-protected L-ornithine NCA, along with L-phenylalanine NCA to yield cationic amphiphilic statistical copolymers in a 4:1 ratio of L-ornithine to L-phenylalanine (see Supporting Information Figs. S4, S5 and Table S3). This ratio was chosen to mimic the hydrophilic to hydrophobic ratio used in the synthesis of poly(vinyl ether) polymers in the preparation of dynamic polyconjugates by Rozema *et al.* to enhance the polymer's ability for endosomal escape (Rozema et al., 2007). Organic GPC analysis showed that the protected polymers had a narrow molecular weight distribution (Table 2, PDI < 1.2) and ^{1}H NMR analysis allowed for the determination of the final monomer composition. Aqueous GPC of the deprotected polymers confirmed the molecular weights of the polymer series, as well as the narrow PDI (Fig. S6). The polymers were also analyzed for weight percent purity as a function of TFA content. In these experiments, the purity was determined as a function of measured nitrogen content and then back calculated to determine the mass of polymer in a sample. Any additional mass was attributed to the trifluoroacetic salt component of the polymer. The obtained percent purity was used as a correction factor in any further experiments.

3.2. siRNA delivery

In this study, the effect of molecular weight or polymer chain length of linear statistical copolymers of L-ornithine and L-phenylalanine was examined. Based on previous work by Rozema *et al.*, all polymer conjugates were prepared at a 5:1 w/w polymer:siRNA where the siRNA was covalently bound to the polymer backbone via a reversible disulfide bond (Rozema et al., 2007). It is assumed that once these polymer conjugates reach the target cell of interest, the disulfide is cleaved intracellularly, releasing the siRNA cargo. Once the siRNA was added to the polyconjugate, the remaining available amines were masked with CDM-GalNAc and CDM-PEG in a 1:3 w/w GalNAc:PEG ratio. These masking groups were utilized as pH-sensitive moieties that remain on the polyconjugate throughout *in vivo* circulation (pH ~7.4), but demask from the polymer conjugates once they have reached the acidic endosomal compartment (pH #16 12#6). It was important that the polymer conjugates were kept at a slightly basic pH in order to ensure that the pendant amine groups remained masked with the CDM groups before dosing. The polymer conjugates were characterized for pH, particle size, siRNA conjugation efficiencies, masking efficiencies, and measured polymer:siRNA ratio (Table 3). In order to leverage the chemical control of this living polymerization to create clear structure-activity relationships, we chose to adopt rigorous analytical characterization and purification for each conjugate. The availability of measured conjugation efficiency, masking efficiency, and polymer:siRNA ratio enables clear conclusions to be drawn from the *in vivo* data.

Further, the polymer conjugates were dosed into rats to investigate their potential for siRNA delivery and mRNA knockdown in liver. From this work, it is clear that an increase in molecular weight leads to increased potency from polymer conjugates prepared from statistical copolymers of L-ornithine and L-phenylalanine (in a 4:1 ratio) (Fig. 2, Table 4). Also, the efficacy of mRNA knockdown shows a dose-dependent potency gain with increasing molecular weight as the siRNA dose of 18 mg/kg showed greater mRNA knockdown than 3 mg/kg, which in turn showed greater mRNA knockdown than 1 mg/kg. While small differences are noted in the measured polymer:siRNA ratios between conjugates, the range of doses and dose response clearly support a relationship between molecular weight and *in vivo* mRNA knockdown. From Fig. 2, it can be seen that at a dose of 3 mg/kg polymer conjugates with a number-average molecular weight of 24.6 kDa (polymer:siRNA = 7.9) have an mRNA knockdown greater than that of a polyconjugate comprised of polymers having a number-average molecular weight of 11.5 kDa (polymer:siRNA of 5.9) at 18 mg/kg.

Furthermore, it should be noted that none of the polymer conjugates tested showed any signs adverse physical effects at doses of 1 or 3 mg/kg, nor did they show any signs of liver damage, relative to a buffer control (Fig. 3). Clinical biochemistry was performed on the blood of the rats tested, and alanine transaminase (ALT) was the marker used to assess toxicity since it is known

Table 3
Characterization of polymer conjugates prepared from (L-ornithine:L-phenylalanine) statistical copolymers; nd = not determined.

Sample	DP_{ORN}	DP_{PHE}	Projected M_n after deprotection (g/mol)	pH	Particle size (radius, nm)	Measured polymer:siRNA Ratio (w/w)	siRNA conjugation efficiency (%)	Masking efficiency (%)
1	77	18	11,500	8.3 ± 0.1	23 ± 2	5.9 ± 0.6	98 ± 1	50 ± 3
2	112	30	17,200	8.5 ± 0.1	nd	nd	82 ± 0.8	nd
3	165	40	24,600	8.3 ± 0.1	23 ± 2	7.9 ± 0.8	98 ± 1	47 ± 2
4	258	63	38,600	8.3 ± 0.1	25 ± 3	7.8 ± 0.8	97 ± 1	48 ± 3

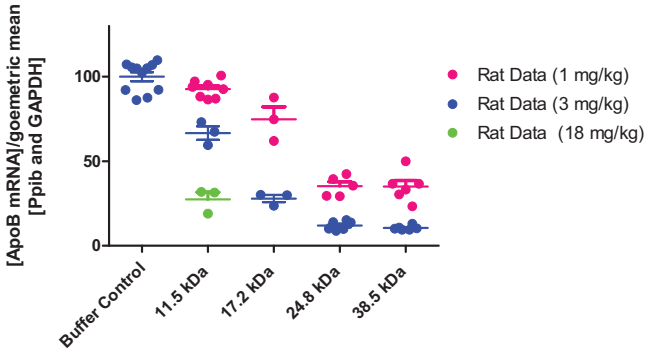


Fig. 2. mRNA knockdown data of poly(L-ornithine:L-phenylalanine) statistical copolymers as a function of molecular weight. Data are expressed as the mean ± SEM (each point represents the ApoB mRNA levels of an individual animal).

to rise dramatically in the case of acute liver damage. Since all groups tested showed no statistical difference in ALT levels at 1, and 3 mg/kg it was assumed that the polymer conjugates tested were safe at these doses. The high dose (18 mg/kg) was used as a means for assessing toxicity of all the polymer conjugates tested. At this high dose, the lowest molecular weight polyconjugate showed no rat deaths, with no observable adverse physical effects in all of the rats dosed with test article. The higher molecular weight polymer conjugates (24.6 kDa or 38.6 kDa) were found to be lethal when tested at 18 mg/kg within 24 h of dosing (with the exception of the 17.2 kDa polyconjugate which was not tested in this toxicity screen), indicating that although the higher molecular weight polymer conjugates were more potent, they were also more toxic. Based on this work, the potential for a defined therapeutic window has emerged for the polymer conjugates having a molecular weight of 24,600 g/mol or 38,600 g/mol, which yield ~70–90% mRNA knockdown data at doses of 1–3 mg/kg with no toxicity observed at those doses.

The increase in mRNA knockdown correlated well with higher levels of siRNA delivered to the livers of the dosed rats as the molecular weight of the polymer increased (Fig. 4). In this assay, whole rat livers were homogenized and examined for siRNA content. From this study, it was clear that polymer conjugates with polymers of higher molecular weight delivered more siRNA to the liver of the rats despite having similar polymer:siRNA (w/w) ratios. The higher liver levels obtained from polymer conjugates of higher molecular weight correlate with the ability of these polymer conjugates to be more potent, as more siRNA was delivered to the hepatocyte target cells.

3.3. Polymer degradability

Since the structure of these polyamide-based polymers is chemically similar to that of a peptide, the potential for these polymers to be biodegradable was assessed using gel electrophoresis in both protease cocktail and lysosomal matrices. In these experiments, a single representative statistical copolymer was fluorophore-labeled with Dylight 650 for detection purposes. The

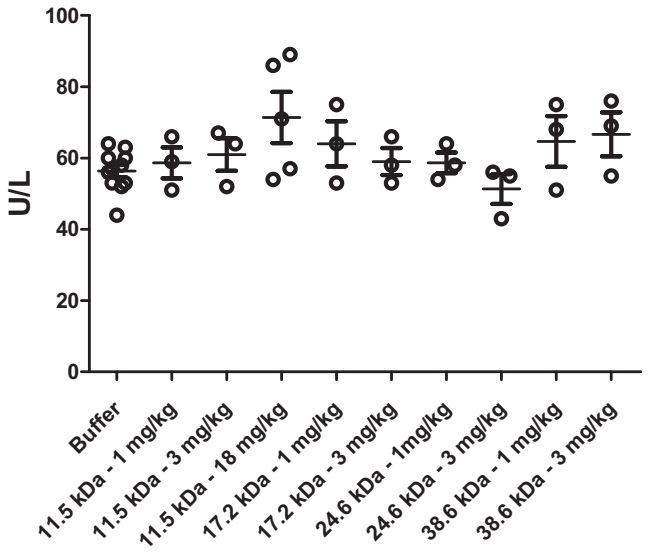


Fig. 3. ALT levels of poly(L-ornithine:L-phenylalanine) statistical copolymers as a function of molecular weight and dose, 48 h post-dose. Data are expressed as the mean ± SEM (each point represents the U/L levels of the individual animals).

bacterial protease cocktail mixture was used as a positive control, since it is a non-specific enzymatic matrix ideal when complete degradation of protein is desired (Burrell, 1993). This cocktail is composed of at least ten proteases, including five serine-type proteases, two zinc endopeptidases, two zinc leucine aminopeptidases and one zinc carboxypeptidase (Burrell, 1993). In the degradation experiments involving the protease cocktail matrix, near complete degradation of the fluorophore-labeled polymer was observed after 2 h (Fig. 5). It was hypothesized that our polyconjugates were endocytosed and trafficked to lysosomes during the normal course of siRNA delivery, so the lysosomal

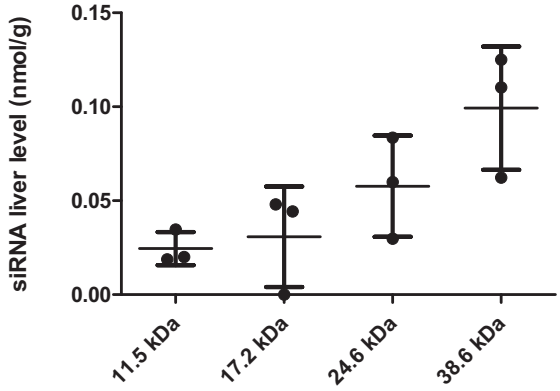


Fig. 4. siRNA liver levels of poly(L-ornithine:L-phenylalanine) statistical copolymers as a function of molecular weight, 48 h post-dose all dosed at 3 mg/kg. Data are expressed as the mean ± SEM (each point represents the siRNA levels in the liver of the individual animals).

Table 4
mRNA knockdown data of poly(L-ornithine:L-phenylalanine) statistical copolymers as a function of molecular weight; nd = not determined; n/a = not applicable.

Sample	Projected M_n after deprotection (g/mol)	mRNA knockdown at 1 mg/kg	mRNA knockdown at 3 mg/kg	mRNA knockdown at 18 mg/kg
1	11,500	7.3 ± 5.1	33 ± 7	73 ± 7
2	17,200	25 ± 13	72 ± 4	nd
3	24,600	65 ± 6	88 ± 3	n/a
4	38,600	65 ± 9	89 ± 1	n/a

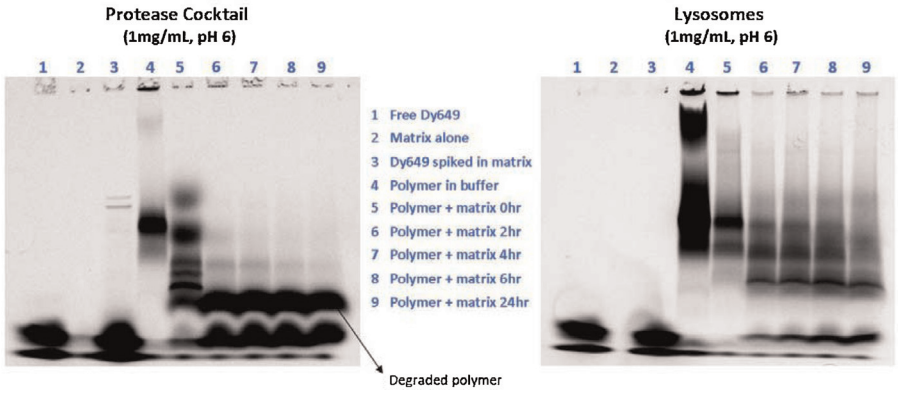


Fig. 5. A time course degradation study of a fluorescent poly(L-ornithine:L-phenylalanine) statistical copolymer using gel electrophoresis.

Fig. 6. Cartoon depiction of the effect of molecular weight on siRNA delivery.

lysates were used as a matrix with more physiological relevance to determine what kind of degradation may occur *in vivo*. The fluorophore-labeled polymer did show bands consistent with degradation products after 2 h in the lysosomal matrix. However, we found that the polymer degradation appeared to be less efficient in the lysosomal matrix when compared to the protease cocktail matrix. This finding is consistent with the fact that at a constant concentration of protein in the matrices (1 mg/mL as measured by BCA assay), the protease cocktail contains 100% proteases whereas only a fraction of the protein within the lysosomal lysates is actually proteases. Taken together, these data show there is a high probability that these copolymers are degraded during the process of delivering siRNA *in vivo*.

The findings of this study show a clear advantage in mRNA knockdown with polyconjugates composed of increased molecular weight, even when holding the polymer:siRNA (w/w) ratio constant (Fig. 6). We have shown that the statistical polymers used herein have the potential for enzymatic degradation. We hypothesize that this potential for degradation may provide an explanation for our observation that *in vivo* efficacy is dependent on polymer molecular weight or polymer chain length. This hypothesis assumes that polymers of increasing molecular weight are more efficient at promoting endosomal escape of siRNA and that as the polymer degrades (shortens) it is less competent for delivery. We view the biodegradability of the polymer backbone as an advantage for this delivery system and expect that it contributes to the defined therapeutic window observed in rodents. It is clear that for these polymer-based siRNA systems, a balance must be met between molecular weight based efficacy, toxicity and degradation.

4. Conclusion

In summary, polymer conjugates were prepared of biodegradable statistical poly(peptides) composed of a 4:1 ratio of L-ornithine and L-phenylalanine at a range of molecular weights, in an attempt to decipher the effect of molecular weight or polymer chain length on siRNA delivery. Using polymers prepared via a living polymerization enabled the synthesis molecular weight controlled polymers with a low polydispersity index to facilitate this comparison. A robust suite of analytical assays and purification of the final conjugates further strengthens the clarity of these results. It was found that polymer conjugates show an increase in efficacy *in vivo* with an increase in molecular weight when held at the same polymer:siRNA ratio. In accordance with increasing potency, these polymer conjugates also show an increase in their ability to deliver siRNA to the liver of the animals with increasing molecular weight. In addition, the toxicity of the resulting polymer conjugates appears to increase as the molecular weight of the polymer increases, but no toxicity was observed at 1–3 mg/kg when >90% mRNA knockdown was achieved suggesting the potential for a defined therapeutic index with these polymer conjugates. The biodegradability potential of these polymer conjugates was shown with both protease cocktail and lysosomal matrices. This work proves the utility of biodegradable polymer conjugates as delivery vehicles for siRNA. It is hypothesized that this type of system could be broadly utilized for siRNA delivery to target liver-specific clinically relevant indications. Future studies will continue to leverage the incorporation of poly(amides) into siRNA delivery systems.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.12.001.

References

Aliferis, T., Iatrou, H., Hadjichristidis, N., 2004. Living Polypeptides. Biomacromolecules 5, 1653–1656.

Bagshaw, R.D., Pasternak, S.H., Mahuran, D.J., Callahan, J.W., 2003. Nicastrin is a resident lysosomal membrane protein. Biochemical and Biophysical Research Communications 300, 615–618.

Berte, L.M., Callihan, D.R., Carlson, J., Charlton, B.J., Flaherty, C.D., Hopper, M.H., Kirkley, B., Ndimbie, O., Schiffgens, J., Stupca, P.J., Sudderth, N., Wilson, J.I., Woodcock, S.M., 2006. Laboratory Documents: Development and Control; Approved Guideline. fifth ed. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Burrell M.M. (Ed.), 1993. Enzymes of Molecular Biology (Methods in Molec. Biol.), pp. 271–276.

Convertine, A.J., Benoit, D.S.W., Duvall, C.L., Hoffman, A.S., Stayton, P.S., 2009. Development of a novel endosomolytic diblock copolymer for siRNA delivery. Journal of Controlled Release 133, 221–229.

Deming, T.J., 2000. Living polymerization of α-amino acid-*N*-carboxyanhydrides. Journal of Polymer Science Part A: Polymer Chemistry 38, 3011–3018.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., Kissel, T., 2003. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 24, 1121–1131.

Gaspar, R.r., Duncan, R., 2009. Polymeric carriers: preclinical safety and the regulatory implications for design and development of polymer therapeutics. Advanced Drug Delivery Reviews 61, 1220–1231.

Habraken, G.J.M., Wilsens, K.H.R.M., Koning, C.E., Heise, A., 2011. Optimization of *N*-carboxyanhydride (NCA) polymerization by variation of reaction temperature and pressure. Polymer Chemistry 2, 1322–1330.

Hadjichristidis, N., Iatrou, H., Pitsikalis, M., Sakellariou, G., 2009. Synthesis of well-defined polypeptide-based materials via the ring-opening polymerization of α-amino acid *N*-carboxyanhydrides. Chemical Reviews 109, 5528–5578.

Itaka, K., Kanayama, N., Nishiyama, N., Jang, W.-D., Yamasaki, Y., Nakamura, K., Kawaguchi, H., Kataoka, K., 2004. Supramolecular nanocarrier of siRNA from PEG-based block catiomer carrying diamine side chain with distinctive pKa directed to enhance intracellular gene silencing. Journal of the American Chemical Society 126, 13612–13613.

Kichler, A., 2004. Gene transfer with modified polyethylenimines. The Journal of Gene Medicine 6, S3–S10.

Kircheis, R., Wightman, L., Wagner, E., 2001. Design and gene delivery activity of modified polyethylenimines. Advanced Drug Delivery Reviews 53, 341–358.

Kowtoniuk, R.A., Pei, T., DeAngelo, C.M., Waldman, J.H., Guidry, E.N., Williams, J.M., Garbaccio, R.M., Barrett, S.E., 2014. Optimization of an α-(amino acid)-*N*-carboxyanhydride polymerization using the high vacuum technique: examining the effects of monomer concentration, polymerization kinetics, polymer molecular weight, and monomer purity. Journal of Polymer Science, Part A: Polymer Chemistry. doi:10.1002/pola.27132.

Matsumoto, S., Christie, R.J., Nishiyama, N., Miyata, K., Ishii, A., Oba, M., Koyama, H., Yamasaki, Y., Kataoka, K., 2008. Environment-responsive block copolymer micelles with a disulfide cross-linked core for enhanced siRNA delivery. Biomacromolecules 10, 119–127.

Morille, M., Passirani, C., Vonarbourg, A., Clavreul, A., Benoit, J.-P., 2008. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials 29, 3477–3496.

Pickel, D.L., Politakos, N., Avgeropoulos, A., Messman, J.M., 2009. A mechanistic study of α-(amino acid)-*N*-carboxyanhydride polymerization: comparing initiation and termination events in high-vacuum and traditional polymerization techniques. Macromolecules 42, 7781–7788.

Rozema, D.B., Lewis, D.L., Wakefield, D.H., Wong, S.C., Klein, J.J., Roesch, P.L., Bertin, S. L., Reppen, T.W., Chu, Q., Blokhin, A.V., Hagstrom, J.E., Wolff, J.A., 2007. Dynamic polyconjugates for targeted in vivo delivery of siRNA to hepatocytes. Proceedings of the National Academy of Sciences 104, 12982–12987.

Stanton, M.G., Colletti, S.L., 2010. Medicinal chemistry of siRNA delivery. Journal of Medicinal Chemistry 53, 7887–7901.

Sun, H., Meng, F., Dias, A.A., Hendriks, M., Feijen, J., Zhong, Z., 2011. α-Amino acid containing degradable polymers as functional biomaterials: rational design, synthetic pathway, and biomedical applications. Biomacromolecules 12, 1937–1955.

Vayaboury, W., Giani, O., Cottet, H., Deratani, A., Schué, F., 2004. Living polymerization of α-amino acid *N*-carboxyanhydrides (NCA) upon decreasing the reaction temperature. Macromolecular Rapid Communications 25, 1221–1224.

Wang, X.-L., Ramusovic, S., Nguyen, T., Lu, Z.-R., 2007. Novel polymerizable surfactants with pH-sensitive amphiphilicity and cell membrane disruption for efficient siRNA delivery. Bioconjugate Chemistry 18, 2169–2177.

Wang, X.-L., Nguyen, T., Gillespie, D., Jensen, R., Lu, Z.-R., 2008. A multifunctional and reversibly polymerizable carrier for efficient siRNA delivery. Biomaterials 29, 15–22.

Weber, A.L., Miller, S.L., 1981. Reasons for the occurrence of the twenty coded protein amino acids. Journal of Molecular Evolution 17, 273–284.

Whitehead, K.A., Langer, R., Anderson, D.G., 2009. Knocking down barriers: advances in siRNA delivery. Nature Reviews Drug Discovery 8, 129–138.

Zimmermann, T.S., Lee, A.C.H., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M.N., Harborth, J., Heyes, J.A., Jeffs, L.B., John, M., Judge, A.D., Lam, K., McClintock, K., Nechev, L.V., Palmer, L.R., Racie, T., Röhl, I., Seiffert, S., Shanmugam, S., Sood, V., Soutschek, J., Toudjarska, I., Wheat, A.J., Yaworski, E., Zedalis, W., Koteliansky, V., Manoharan, M., Vornlocher, H.-P., MacLachlan, I., 2006. RNAi-mediated gene silencing in non-human primates. Nature 441, 111–114.