Journal of Controlled Release 209 (2015) 57–66

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Protease-triggered siRNA delivery vehicles

David B. Rozema [a,*], Andrei V. Blokhin [a], Darren H. Wakefield [a], Jonathan D. Benson [a], Jeffrey C. Carlson [a], Jason J. Klein [a], Lauren J. Almeida [a], Anthony L. Nicholas [a], Holly L. Hamilton [a], Qili Chu [a], Julia O. Hegge [a], So C. Wong [a], Vladimir S. Trubetskoy [a], Collin M. Hagen [a], Eric Kitas [b], Jon A. Wolff [a], David L. Lewis [a]

[a] Arrowhead Research Corporation, Arrowhead Madison, 465 Science Drive, Madison, WI 53711, USA
[b] F. Hoffmann-La Roche Ltd., Pharma Research and Early Development Discovery Chemistry, Roche Innovation Center Basel, Grenzacherstrasse 124, CH-4070 Basel, Switzerland.

ARTICLE INFO

Article history:
Received 2 January 2015
Received in revised form 5 April 2015
Accepted 11 April 2015
Available online 14 April 2015

Keywords:
Targeted delivery
siRNA polymer conjugate
Subcutaneous

ABSTRACT

The safe and efficacious delivery of membrane impermeable therapeutics requires cytoplasmic access without the toxicity of nonspecific cytoplasmic membrane lysis. We have developed a mechanism for control of cytoplasmic release which utilizes endogenous proteases as a trigger and results in functional delivery of small interfering RNA (siRNA). The delivery approach is based on reversible inhibition of membrane disruptive polymers with protease-sensitive substrates. Proteolytic hydrolysis upon endocytosis restores the membrane destabilizing activity of the polymers thereby allowing cytoplasmic access of the co-delivered siRNA. Protease-sensitive polymer masking reagents derived from polyethylene glycol (PEG), which inhibit membrane interactions, and N-acetylgalactosamine, which targets asialoglycoprotein receptors on hepatocytes, were synthesized and used to formulate masked polymer-siRNA delivery vehicles. The size, charge and stability of the vehicles enable functional delivery of siRNA after subcutaneous administration and, with modification of the targeting ligand, have the potential for extrahepatic targeting.

1. Introduction

Competing attributes must be included in the design of any systemically administered, selectively-targeted delivery vehicle for membrane impermeable therapeutics: interactions with membranes that facilitate cytoplasmic delivery and an absence of interactions with membranes, which is necessary for cell targeting and safety. Membrane perturbation is required in order to facilitate the transit of membrane impermeable material such as siRNA into the cytoplasm of the cell. However, uncontrolled, non-specific membrane interactions reduce circulation, preclude cell targeting, and are inherently cytotoxic. Due to these non-specific interactions, it is necessary to control the membrane permeabilizing ability of delivery vehicles such that membrane perturbation occurs only at the desired location. The incorporation of a physiologically-sensitive triggering mechanism to switch between membrane disruptive and membrane inert states is necessary for a successful delivery vehicle.

Acid-labile functional groups, which rely upon the pH gradient present in the endosome/lysosome pathway, have been incorporated as triggers to regulate the membrane lytic activity of delivery vehicles. Labile bonds such as acetals [1–3], hydrazone [4], ortho esters [5–8], vinyl ethers [9] and maleamates [10–12] have been incorporated into liposomes and amphipathic polymers to control interactions with membranes and provide a trigger for cytoplasmic delivery.

In addition to the pH gradient that exists in the endosome/lysosome pathway, the increase in hydrolytic enzymes, nucleases, esterases and proteases has also been harnessed to great utility in the targeted delivery of small molecule prodrugs [13]. The use of enzymatic triggers is attractive in that the hydrolyzed bonds (e.g. amides, esters, phosphodiesters) are relatively easily synthesized and stable in the absence of degrading enzymes. Despite the attractiveness of enzyme-based triggering mechanisms there is a paucity of examples using lysosomal enzymes to trigger the release of macromolecular therapeutics. The most obvious reason for this lack of hydrolase-triggered delivery is the fact that the macromolecular therapeutic (either protein or polynucleic acid) is itself a substrate for hydrolysis, and as a consequence hydrolase-triggered cytoplasmic release of these agents is in competition with the degradation of the therapeutic itself. The advent of nuclease-resistant oligonucleotides as therapeutic agents has provided an opportunity for the design of nucleic acid delivery vehicles that rely on hydrolytic enzymes for triggered cytoplasmic release of nucleic acids.

Presented herein is the development of protease-sensitive delivery vehicles for siRNA. These delivery vehicles expand on the pH-sensitive dynamic polyconjugates (DPCs) previously described [14]. The pH-sensitive DPCs utilized a maleic anhydride for reversible masking of the membrane-interactive polyamine. The maleic anhydride and amine form a maleamate that is stable under basic conditions, but revert

* Corresponding author.
E-mail address: dave.rozema@arrowres.com (D.B. Rozema).

http://dx.doi.org/10.1016/j.jconrel.2015.04.012

back to anhydride and membrane-interactive polyamine (R-NH_2 in Fig. 1) under acidic physiological conditions. The more stable protease-sensitive linkages survive longer in circulation, and allow an increased range of targeting using the same polymers as those developed for pH-sensitive DPCs.

2. Materials and methods

2.1. Materials

2,2′-Azobis(2-methylpropionitrile) (AIBN, radical initiator), 2-(2-aminoethoxy) ethanol, BOC anhydride, acryloyl chloride and 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPCPA, RAFT Agent) were purchased from Sigma Aldrich. Sec-butyl acrylate monomer was obtained from Polysciences Inc. 12 units PEGylation reagent (m-dPEG12-NHS) was purchased from Quanta Biodesign.

2.1.1. siRNAs

The siRNAs of the following sequences were synthesized using standard phosphoramidite chemistry.

siF7 sense: 5′-(NH_2–C6)-GfcAfaAfgGfcGfuGfcCfaAfcUfcAf (invdT)
siF7 antisense: 5′dT*GfaGfuUfgGfcAfcGfcCfuUfuGfcdT*dT-3′
siLuc sense: 5′-(NH_2–C6)-cuuAcGcuGAGuAcuucGAdT*dT-3′; and
siLuc antisense: 5′-UCGAAGuACUcAGCGuAAGdT*dT-3′.

(lower case, 2′-OMe substitution; f, 2′-F substitution; d, 2-deoxy substitution; and *, phosphorothioate linkage.)

2.2. Modifications of amine siRNA with acetyl-protected thiol group

SATA-modified siRNAs were synthesized by reaction of 5′ amine-modified siRNA with 1 weight equivalents (wt. eq.) of N-succinimidyl-S-acetylthioacetate (SATA) reagent (Pierce) and 0.36 wt. eq. of $NaHCO_3$ in water at 4 °C for 16 h. The modified siRNAs were then precipitated by the addition of 9 volumes of ethanol and incubation at −80 °C for 2 h. The precipitate was dissolved in RNase-free PBS buffer and quantified by measuring absorbance at the 260 nm.

2.3. N-Boc-ethoxyethylamine acrylate

In a 2L round-bottom flask equipped with a stir bar, 2-(2-aminoethoxy) ethanol (21.1 g, 202.9 mmol, Sigma Aldrich) was dissolved in 350 mL dichloromethane. In a separate 1L flask, BOC anhydride (36.6 g, 169.1 mmol) was dissolved in 660 mL dichloromethane. The 2L round-bottom flask was fitted with an addition funnel and BOC anhydride solution was added to the flask over 6 h. The reaction was left to stir overnight. In a 2L separatory funnel, the product was washed with 300 mL each of 10% citric acid, 10% K_2CO_3, sat. $NaHCO_3$, and sat. NaCl. The product, BOC protected 2-(2-aminoethoxy) ethanol, was dried over Na_2SO_4, gravity filtered, and DCM was evaporated using rotary evaporation and high vacuum.

In a 500 mL round bottom flask equipped with a stir bar and flushed with argon, BOC protected 2-(2-aminoethoxy) ethanol (27.836 g, 135.8 mmol) was added, followed by 240 mL anhydrous dichloromethane. Diisopropylethyl amine (35.5 mL, 203.7 mmol) was added, and the system was placed in a dry ice/acetone bath. Acryloyl chloride (12.1 mL, 149.4 mmol) was diluted using 10 mL of dichloromethane, and added drop-wise to the argon flushed system. The system was kept under argon and left to come to room temperature and stirred overnight. The product was washed with 100 mL each of dH2O, 10% citric acid, 10% K_2CO_3, sat. $NaHCO_3$, and saturated NaCl. The product, BOC-amino ethyl ethoxy acrylate (BAEEA), was dried over Na_2SO_4, gravity filtered, and DCM was evaporated using rotary evaporation. The product was purified through column chromatography on 29 cm of silica using a 7.5 cm diameter column. The solvent system used was 30% ethyl acetate in hexane. Rf: 0.30. Fractions were collected and solvent was removed using rotary evaporation and high vacuum. Boc-ethoxyethylamine acrylate was obtained with 74% yield and was stored in the freezer.

2.4. RAFT copolymer of N-Boc-ethoxyethylamine acrylate and sec-butyl acrylate (EAB)

Solutions of AIBN (1.00 mg/mL) and RAFT agent CPCPA (10.0 mg/mL) in butyl acetate were prepared. Monomer molar feed was 55% *N*-Boc-ethoxyethylamine acrylate, 45% sec-butyl acrylate (CAS # 2998-08-5). Theoretical Mw was 100,000.

N-Boc-ethoxyethylamine acrylate (0.890 g, 3.43 mmol) sec-butyl acrylate (0.391 mL, 0.360 g, 2.81 mmol) CPCPA solution (0.350 mL, 0.0125 mmol), AIBN solution (0.308 mL, 0.00188 mmol), and butyl acetate (5.3 mL) were added to a 20 mL glass vial with a stir bar. The vial was sealed with a septa cap and the solution bubbled with nitrogen using a long syringe with a second syringe as the outlet for 1 h. The syringes were removed and the vial heated to 80 °C for 16 h using an oil bath. The solution was allowed to cool to room temperature and transferred to a 50 mL centrifuge tube before hexane (35 mL) was added to the solution. The solution was centrifuged for 2 min at 4400 rpm. The supernatant layer was carefully decanted and the bottom (solid or gel-like) layer was rinsed with hexane. The bottom layer was then re-dissolved in DCM (7 mL), precipitated in hexane (40 mL) and centrifuged once more. The supernatant was decanted and the bottom layer rinsed with hexane before the polymer was dried under reduced pressure for several hours. The yield of crude EAB copolymer was 0.856 g. Samples of the crude polymer were taken for multi-angle light scattering (MALS). The dried, crude copolymer was dissolved in DCM (100 mg/mL). Hexane was added until just after the cloud point was reached. The resulting milky solution was centrifuged. The bottom layer was extracted and fully precipitated into hexane. The fraction was centrifuged, after which the copolymer was isolated and dried under vacuum. The yield of isolated fraction of EAB copolymer was 0.478 g. Samples of the fractionated copolymer were taken for ^{1}H-NMR and MALS. The composition determined by ^{1}H-NMR was 61% *N*-Boc-ethoxyethylamine and acrylate, 39% sec-butyl acrylate.

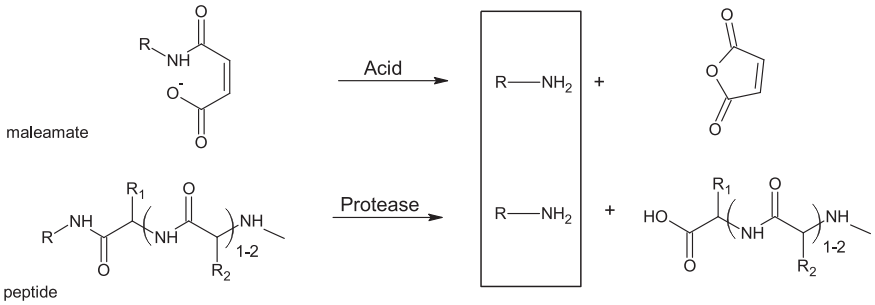


Fig. 1. The common amine product of pH-labile maleamate and protease cleavable amide bonds.

2.5. MALS analysis

Approximately 10 mg of the copolymer was dissolved in 0.5 mL 75% dichloromethane, 20% tetrahydrofuran, and 5% acetonitrile. The molecular weight and polydispersity (PDI) were measured using a Wyatt Heleos II multiangle light scattering detector attached to a Shimadzu Prominence HPLC using a Jordi 5 μ 7.8 × 300 Mixed Bed LS DVB column. Crude Polymer: MW: 59,000 (PDI 1.3), Fractionated Polymer: MW 70,000 (PDI:1.1).

2.6. Deprotection/dialysis

The dried samples were treated with 2 M HCl in acetic acid (~7 mL) for 1 h to remove the BOC protecting groups. Then the reaction was diluted with 20 mL of water and allowed to stir for 10–15 min. The fractions were then dialyzed with 3500 MW dialysis tubing in high salt, and water for 15 h, 8 h, and 15 h respectively. The fractions were then transferred to 50 mL centrifuge tubes and lyophilized for 3 days or until dry. The dry samples were brought up at 20 mg/mL in water for further study.

2.7. Small molecule syntheses

Protease masking groups PEG-FCit-PABC, PEG-fcit PABC, NAG-ACit PABC and NAG-acit PABC were synthesized using standard amide coupling reagents. See supplemental data for synthetic details.

2.8. Hemolysis

Lysis of rat red blood cells (RBCs) was assessed by hemoglobin release assay. RBCs (1 mL) were diluted into 1× PBS (Corning cellgro) and rinsed 5 times by centrifugation (5 min at 2000 rpm). The rinsed cells were further diluted to give a 2% final concentration RBC solution. To each well (96 well NUNC round bottom plate) 75 μL of 1× PBS was added, to the first well an additional 63 μL of PBS was added as well as 12 μL of modified polymer (5 mg/mL) and serial dilutions were performed, discarding the last 75 μL. The assay plate was incubated at room temperature for 1 h. After 1 h incubation, the plate was centrifuged at 1000 rpm for 5 min and supernatant (25 μL) from each well was diluted within PBS buffer (175 μL) in a 96 well flat-bottomed plate (NUNC). Hemolysis was monitored by measuring the absorbance of the release hemoglobin at 405 nm using a plate reader (TECAN infinite M1000 Pro). Hemolysis was determined relative to the positive lysis control Triton X-100 (10% v/v in water) and the negative control buffer. The percentage of hemolysis was calculated as: 1 − ((Absorbance sample − Absorbance negative control) / (Absorbance of positive control − Absorbance negative control)). HC_{25} was defined as the polymer concentration causing 25% hemolysis. The experiments were done in triplicate.

2.9. TNBS assay

2,4,6 trinitrobenzene sulfonic acid (TNBS, Eastman Kodak) was dissolved in water at 2 mg/mL. To each well (96 well flat-bottom plate NUNC) was added 190 μL of 50 mM BORAX pH 9 (sodium tetraborate decahydrate, Aldrich) and 8 μL of TNBS (2 mg/mL). Unmodified polymer at 2 mg/mL was added to give the desired standard curve (0–12 μg). 10 μg of modified polymer was added and incubated at room temperature for 20 min. Absorbance was measured at 420 nm using TECAN (infinite M100Pro). % modification values were calculated from the unmodified polymer standard curve. Experiments were performed in triplicate.

2.10. siRNA-polymer formulations

Polyacrylate EAB in 5 mM pH 8.0 HEPES buffer was modified 1.5 wt.% with the activated disulfide reagent succinimidyloxycarbonyl-alpha-methyl-alpha(2-pyridyl-dithio)toluene (SMPT) (Pierce) to provide thiol reactive groups for subsequent attachment of siRNA. The thiol-reactive polymer was then diluted to 5 mg/mL in 60 mg/mL HEPES base. To this solution was added 10 mg/mL various protease-cleavable PEG masking reagents. This amount of amine-reactive PEG reagents is 0.25 molar equivalents to polymer amine groups. After 1 h, acetyl-protected thiol endogenous rodent or primate factor VII siRNA (0.1 to 0.2 wt. eq relative to polymer) was added to the polymer solution. After incubation overnight, conjugates were further modified by the addition of an N-acetylgalactosamine derivative CDM-NAG (presented in Fig. 6) or NAG-ACit-PABC (presented in Figs. 7–9), which was added to 30 mg/mL (2 mol eq per amine for CDM-NAG and 1 mol eq per polymer amine for PABC reagents) and incubated for 30 min.

2.11. Mice and injection procedures

Female ICR mice, 6 to 8 weeks old, were obtained from Harlan Sprague–Dawley, (Indianapolis, IN). All the mice were handled in accordance with animal used protocols approved by the Animal Care and Use Committee at Arrowhead Madison Inc. Mice were maintained on a 12 h light/dark cycle with free access to water and food (Harlan Teklad Rodent Diet, Harlan, Madison, WI). For mouse injections, modified polymer and siRNA were mixed together in delivery solution and injected as a bolus into the tail vein in a total volume of 0.2 mL of HEPES-buffered (5 mM, pH 7.5) isotonic glucose under standard conditions. For the subcutaneous injections, an area of loose skin on the back is tented and a needle (22 to 25 gauge) is inserted just under the skin in the dorsal aspect of the neck with similar formulation conditions.

2.12. Confocal imaging of fluorescently-labeled polymer

For the preparation of fluorescently tagged EAB, Cy3 NHS ester (GE Healthcare, Piscataway,NJ) was coupled to EAB according to the manufacturer's recommendations. 100 μg Cy3-labeled EAB that was modified with PEG-FCit-PABC and NAG-ACit-PABC or PEG-FCit-PABC alone was injected into the tail vein of mice. Liver tissues were harvested 6 h postinjection and preparation of tissue sections were performed as previously described [14]. Tissue sections were counterstained with Alexa-633-conjugated phalloidin (20 nM, Invitrogen, Carlsbad, CA) and To-pro-3 (40 nM, Invitrogen) in PBS as previously described [14]. Mounted slides were analyzed using a Zeiss LSM710 confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY).

2.13. Non-human primate study

Male cynomolgus monkeys were housed at the University of Wisconsin–Madison. Prior to injection, animals were sedated with ketamine (10 mg/kg, injected intramuscularly). DPC samples were injected into the small saphenous vein slowly over 5 min at a volume of 2 mL/kg or subcutaneously in between the shoulder blades over 5-10 s at a volume of 0.1 mL/kg. Blood samples were collected every 3–4 days for measurement of fVII activity and prothrombin time. All procedures were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (National Research Council [U.S.], 2011) and were approved by the University of Wisconsin–Madison and Arrowhead Madison, Inc. animal care and use committees.

2.14. Quantitative Real-Time PCR

In preparation for quantitative PCR, total RNA was isolated from tissue samples homogenized in TriReagent (Molecular Research Center)

following the manufacturer's protocol. Approximately 500 ng RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Pre-manufactured TaqMan gene expression assays for mouse Factor VII (Assay ID: Mm00487333_m1) and ß-actin (Part#: 4352341E) were used in biplex reactions in triplicate using TaqMan Gene Expression Master Mix (Applied Biosystems). Quantitative PCR was performed by using a 7500Fast or StepOnePlus Real-Time PCR system (Applied Biosystems) and analyzed by the $\Delta\Delta C_T$ method to calculate relative gene expression.

2.15. Serum FVII activity measurements

Serum samples form mice were prepared by collecting blood (9 volumes) by submandibular bleeding into microcentrifuge tubes containing 0.109 M sodium citrate anticoagulant (1 volume) following standard procedures. FVII activity in serum is measured with a chromogenic method using a test kit (BIOPHEN VII, Aniara, Mason, OH) following the manufacturer's recommendations. Absorbance of colorimetric development was measured using a Tecan Safire 2 microplate reader at 405 nm.

2.16. Zeta and particle sizing

The size and surface potential of the siRNA-polymer conjugates were measured by light scattering using a Malvern, ZetaPlus Particle Sizer, I90. Samples were analyzed at a polymer concentration of 5 mg/mL polymer (0.5 mg/mL siRNA).

3. Results and discussion

There are three main components in a DPC siRNA delivery formulation: siRNA, a membrane-interactive polymer, and an agent that reversibly masks the membrane interactions of the polymer. A variety of polymers have been studied as components in DPCs, but the common design motif is amphipathicity. Hydrophilic character is derived from primary amines, which serve as sites for reversible masking either by pH-labile maleic anhydride groups [14] or the protease-labile groups described in this study (Fig. 1). Polymers developed for pH-sensitive DPCs have been demonstrated to deliver siRNA to the liver in vivo when their amine groups were reversibly masked with pH-labile hepatocyte targeting groups [14]. In this report, we examined reversible masking of the same amphipathic polymers (R-NH_2 in Fig. 1) with putative protease-labile hepatocyte targeting reagents for their ability to facilitate cytoplasmic access of siRNA.

The membrane interactive polymer used to test the reversibility of protease-cleavable reagents for delivery of siRNA is an amphipathic polyacrylate that is derived from copolymerization of ethoxyethylamine and sec-butyl acrylate monomers to produce a polymer termed EAB (Fig. 2a). Alkyl monomer *sec*-butyl acrylate (45 mol% of feed) and amine monomer *tert*-butoxycarbonyl (2-aminoethoxy)ethyl acrylate (55 mol% of feed) were copolymerized to generate an amphipathic polymer. The amine monomer incorporates at a slightly faster rate, which results in higher amine incorporation than the feed at an average of 40 ± 5% butyl to 60 ± 5% amine. The chemical structure of EAB is presented in Fig. 2a, but is represented in illustration Fig. 5 as a green curve where amine side chains are represented as green ovals and propyl side chains are gray ovals. In separate studies, EAB and similar amphipathic polyacrylates were shown to be effective components in DPC formulations when masked with pH-labile modifications [15]. EAB was polymerized by controlled reversible addition-fragmentation chain-transfer (RAFT) polymerization using 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPCPA) as a RAFT agent. Polymers produced under RAFT conditions are synthesized synchronously such that the resulting polymers are similar in composition [16].

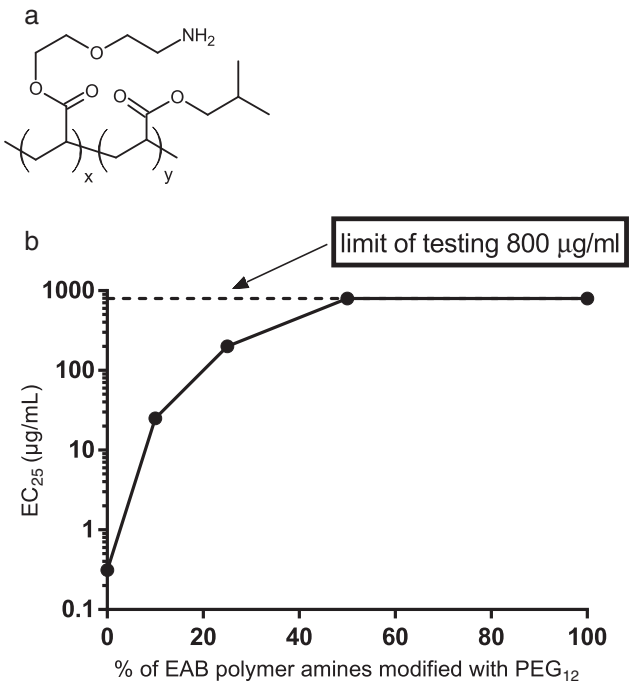


Fig. 2. a) The structure of amphipathic delivery polycation based on co-polymerization of ethoxyethylamine and isobutyl acrylates (EAB). The polymer is generated by random co-polymerization of amine, which is protected with BOC groups during polymerization, and alkyl monomers. b) The effective concentration of EAB required to attain 25% hemolysis (EC_{25}) as a function of modification with PEG_{12}.

The amphipathic polycation EAB interacts with negatively charged membranes by electrostatic and hydrophobic interactions. A measure of the membrane instability caused by these interactions is the amount of hemoglobin released from red blood cells (RBCs) upon exposure to membrane interactive agents [17,18]. EAB at 0.3 µg/mL concentration results in 25% lysis of RBCs while EAB that has greater than >95% of amine groups modified with PEG_{12}-NHS ester has no observable hemolytic activity up to practical limit of testing of 800 µg/mL, which is a greater than 2500-fold reduction in the membrane lytic properties of EAB (Fig. 2b). Even a moderate level of PEG modification results in a significant reduction in hemolytic activity as a 10% PEG modification results in an 80-fold decrease in EC_{25} (from 0.3 to 25 µg/mL). These results demonstrate both the membrane disruptive ability of EAB and the effect of amine modification to attenuate membrane disruption by reduction of charge and increasing hydrophilic composition.

The key to functional in vivo delivery is to control polymer–membrane interactions such that they are reduced until the desired intracellular site of activation is desired. A particularly well-studied protease-cleavable prodrug strategy uses the 1,6 elimination of a *p*-aminobenzyloxycarboxyl (PABC) group to produce a traceless linker to an amine-containing drug (Fig. 3). Upon proteolysis of the anilide bond of amino acid (Aa) PABC derivative, there is an elimination reaction to produce carbon dioxide, an amine, and an azaquinonemethide, which then reacts with water to form *p*-aminobenzyl alcohol [19]. These series of reactions result in the formation of an amine and an amino acid, just as produced in a standard proteolytic reaction; however, the PABC group provides a spacer that allows for proteolysis of non-peptidic amino substrates.

The widely-expressed lysosomal protease cathepsin B (catB) is a commonly-invoked protease for the release of prodrugs, whose preferred substrates have a cationic residue (K or R) followed by a hydrophobic residue (F, V etc.) on the carboxy side of the hydrolyzed peptide bond [20,21]. For example, the majority of the work presented in this report uses the H_2N-phenylalanine-citrulline-PABC-CO_2H (FCit-PABC) motif, where proteolysis is presumed to occur between citrulline

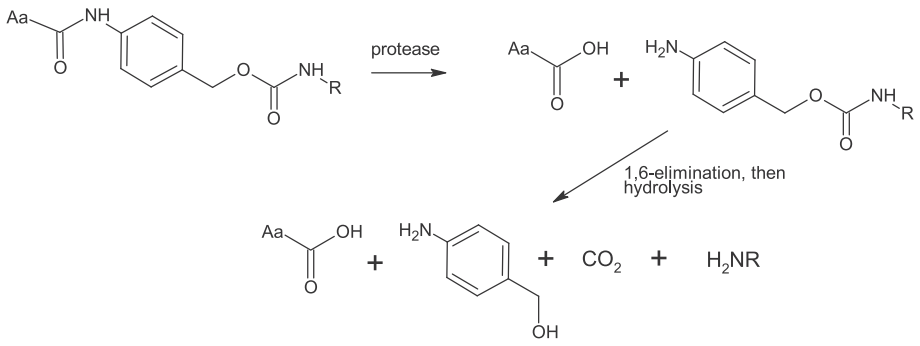
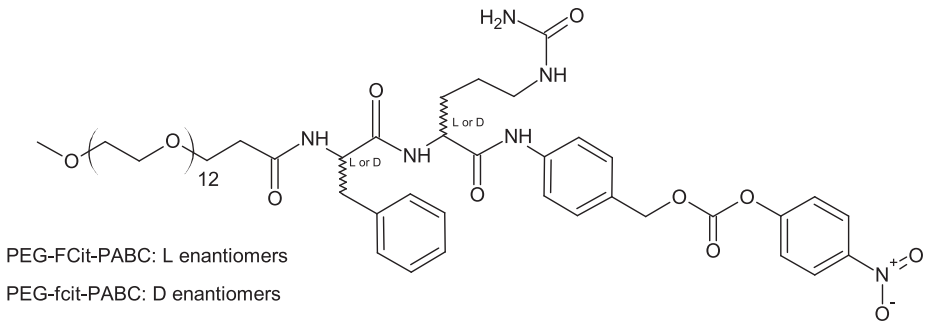


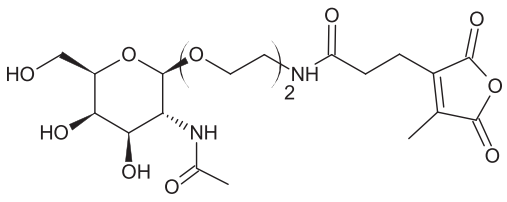
Fig. 3. The structure and cleavage of self-immolating linker based upon *p*-aminobenzyl alcohol.

and PABC. Citrulline is an uncharged isostere of arginine, which due to its neutrality is a particularly attractive amino acid residue for the purposes of circulation and targeting in vivo [20–22].

In DPC formulations, the membrane interactive polymer is reversibly masked with a mixture of polyethylene glycol (PEG) and cell-targeting ligands [14]. PEG groups reduce non-specific interactions and prevent the aggregation of the nucleic acid delivery vehicles. Cell-specific targeting to hepatocytes is accomplished by modification of the delivery vehicle with N-acetylgalactosamine (NAG), which is a ligand for the asialoglycoprotein receptor on hepatocytes [23].

Attachment of PEG groups to a polymer decreases non-specific interactions in vivo [24]; however, permanent PEG masking of >10% of polymer amines completely inhibits intracellular delivery ability of pH-labile DPC formulations (vide infra). For this reason, the ability of a partially PEGylated polymer to deliver functional siRNA is an indirect assessment of the reversibility of the PEG modification: i.e. only reversible





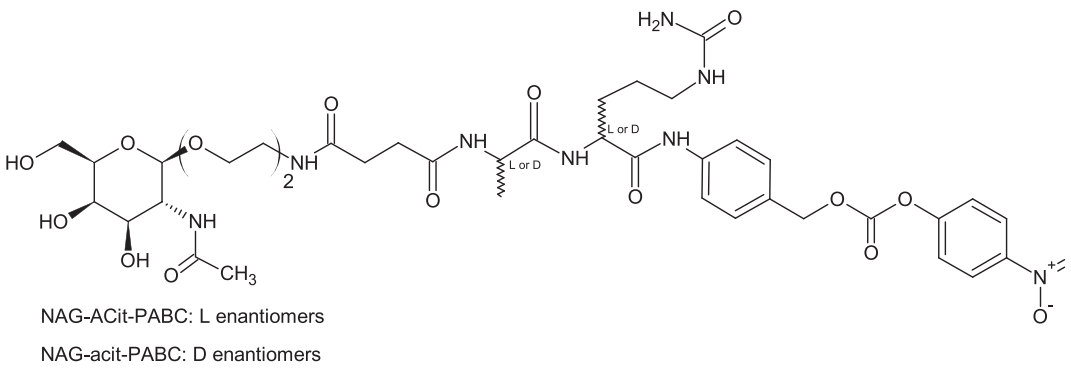


Fig. 4. Reagents used for the modification of polyamine: protease-cleavable reversible masking reagents based upon *p*-aminobenzyl carbonate (PABC derivatives) and pH reversible N-acetylgalactosamine (NAG) reagent based upon disubstituted maleic anhydride (CDM-NAG).

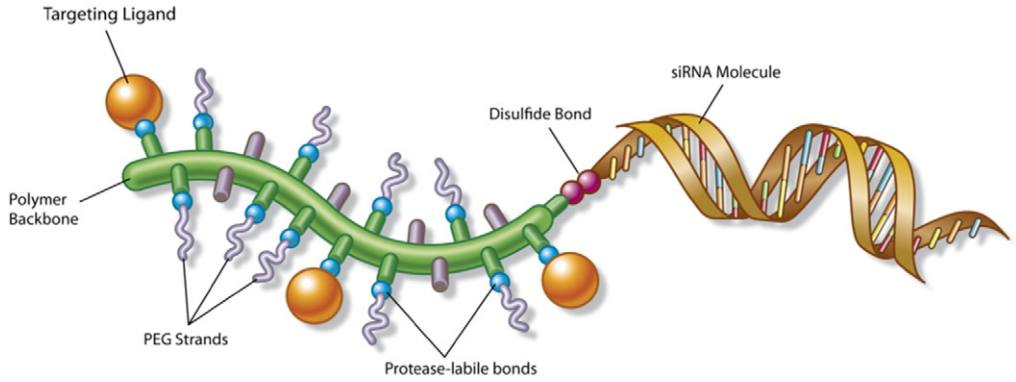


Fig. 5. Illustration of DPC where polymer EAB (green curve with amine side chains represented as green ovals and alkyl groups that are denoted as gray ovals). The amine side chains of EAB are modified with PEG (gray squiggles) or hepatocyte-targeting NAG groups (orange circles) via pH or protease-labile bonds (blue circles). siRNA is attached to the polymer via disulfide bonds (two purple circles). The placement of the disulfide bonds is randomly placed on polymer amine side chains, and not on the end of the polymer as represented.

PEG masking results in functional siRNA delivery while irreversibly-attached PEG completely inhibits the delivery of functional siRNA.

To simplify synthesis and screening of amino acid-based masking reagents, relatively easy to synthesize (PEG) derivatives were initially evaluated. PEG reagents were composed of a discrete 12-mer of PEG and naturally occurring L enantiomers of the amino acid sequence phenylalanine-citrulline (FCit). The PEG masking reagents were activated for reaction with amine groups as *p*-nitrophenol carbonate esters, which react with amine groups to form a carbamate bond (PEG-FCit-PABC).

PEG-modified delivery vehicles are not selectively targeted to any tissues in vivo and require masking with a targeting ligand. Functional delivery of siRNA also requires that the targeting group not inhibit interaction between the delivery polymer and the endosomal membrane. Unfortunately, the valency and hydrophilicity of NAG targeting reduce polymer–membrane interactions. Therefore, NAG attachment to a DPC polymer must be reversible for the functional delivery of siRNA. As an initial test of protease-labile PEG reagents, hepatocyte targeting is accomplished by reversible modification with pH-labile CDM-NAG (Fig. 4), which reversibly modified polyamines with NAG via a pH-labile maleamate group [14]. This pH-labile NAG reagent in combination with PEG reagents whose reversibilities are under evaluation enables testing of the new reversible chemistries without synthesis of relatively complicated N-acetylgalactosamine derivatives. The chemical structures of reagents synthesized for the modification of polyamine EAB are presented in Fig. 4, but are also represented schematically in Fig. 5 where PEG is gray squiggle and NAG is an orange circle. The labile bonds (either protease-labile PABC or pH-labile maleamate bonds) are represented as blue circles.

The formulation of DPC delivery vehicles was a four-step process. At each step the four components of a hepatocyte-targeted DPC are introduced: polymer, PEG, siRNA and NAG. First, the polymer was modified with a pyridyl disulfide group using a commercially-available amine-reactive reagent. Second, the polymer was modified with 0.25 mol equivalents of PEG reagent per mole of amine in the polymer. This PEG to amine stoichiometry resulted in PEG attachment to 20–25% of the polymer amines, which is measured by the reduction in amine content as measured by colorimetric assay using trinitrobenzenesulfonic acid (TNBS). This extent of PEG modification is enough to inhibit the membranolytic activity of the formulation (Fig. 2a and delivery results to follow in Fig. 6), but not extensive enough to significantly inhibit the electrostatic interactions between the cationic polymer and anionic nucleic acids.

Third, thioacetyl-modified siRNA [14] against the endogenous, hepatocyte-expressed blood clotting factor VII (fVII) was added to the formulation. Chemical modification of siRNAs provides protection from rapid degradation in serum by ribonucleases [25] and prevents cytokine induction [26]. For these reasons, the riboses of the siRNA used in this study were modified at the 2′ position with a mixture of methoxy and fluoro groups. As a measurement of functional knockdown, siRNA targeting coagulation Factor 7 (FVII) was used. FVII is expressed exclusively in hepatocytes and secreted into the bloodstream. The level of FVII activity in plasma is easily monitored by a simple chromogenic assay. To test for any potential vehicle-related knockdown, formulations were also made using siRNAs designed for the exogenous firefly luciferase gene.

The anionic siRNA and cationic partially PEGylated polymer form an electrostatic complex. In order to prevent aggregation during the siRNA-polymer conjugation reaction, one of the components of the polyelectrolyte complex (either siRNA or polymer) must be in excess. As a practical matter, the relatively inexpensive reagent (polymer) is added in excess. For data presented in this report, the polymer to siRNA is a 10:1 weight ratio (3:1 molar ratio). The electrostatic complex between polymer and siRNA accelerates the reaction between nucleophilic amine groups of the polymer and electrophilic thioacetyl group of the siRNA, which results in the acetylation of one polymer amine and the formation of a thiol on siRNA. The siRNA thiol may then react with the pyridyl disulfide on the polymer resulting in a siRNA-polymer conjugation via a disulfide bond [14]. The resulting disulfide bond between EAB and siRNA is represented in Fig. 5 as two purple circles.

The efficiency of conjugation of siRNA and polymer was calculated from the ratio of unconjugated siRNA in formulations to the amount of free siRNA after reduction with dithiothreitol. Quantitation of free siRNA was performed using agarose gel electrophoresis and staining

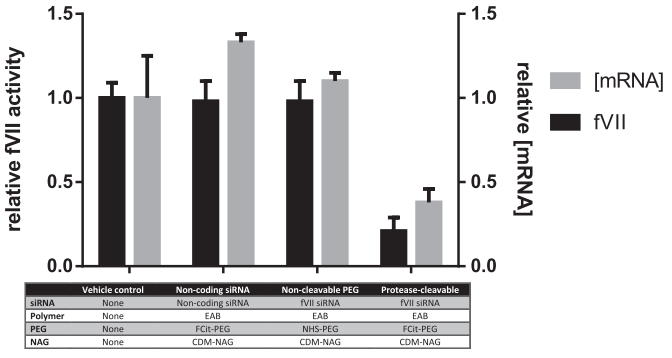


Fig. 6. fVII activity (left axis, black bars) and mRNA quantitation (right axis, gray bars) after intravenous administration of DPC formulations masked with a mixture of pH-labile (CDM-NAG) and protease-labile modifications. Formulations were injected via tail vein in mice (n = 4). Samples were taken 7 days postinjection. fVII activities and mRNA levels were normalized to mice treated with isotonic glucose.

with ethidium bromide [14]. The extent of conjugation is in the range of 80–90% for formulations presented in this report (Table 1).

Once polymer and siRNA were conjugated, the polymer-siRNA conjugate was exhaustively modified with 2 mol equivalents of maleic anhydride derivative CDM-NAG (Fig. 4) per polymer amine, which reacts with the remaining 50% of the polymer amines. The result is a masked, hepatocyte-targeted siRNA-polymer conjugate (DPC). The degree of polymer modification after the addition of excess of maleic anhydride is extensive enough (>95% of available amines) such that there is little if any electrostatic interaction between polymer and siRNA, and there would be no co-targeting of siRNA and polymer without covalent attachment of siRNA to polymer [14,27]. The resulting NAG-modified polymer-sRNA conjugate (illustrated in Fig. 5) is ready for injection.

The formulated vehicles were then injected into the tail vein of mice (20 μg of siRNA conjugated to 200 μg of polymer in 200 μL volume). Serum and liver tissue were collected one week after tail vein injection. The enzymatic activity of the FVII protease was measured in plasma, and the amount of FVII transcript was quantified using real-time PCR after mRNA was extracted from liver tissue. The results in Fig. 6 showed that protease cleavable PEG-modified DPC achieved a 79% knockdown in serum FVII activity (left axis, dark bars) and a corresponding 62% knockdown in FVII mRNA (right axis, light bars).

No FVII knockdown, relative to injection of isotonic glucose, was observed with a DPC containing a non-coding siRNA indicating that FVII knockdown was sequence specific, consistent with an RNAi mechanism. To demonstrate that irreversible PEG modification is truly inhibitory of functional delivery, formulations were permanently PEG modified with a commercially-available PEG_{12} NHS ester. Consistent with protease-mediated unmasking, the formulation with a non-cleavable PEG did not result in knockdown. The only formulation that resulted in knockdown of fVII activity and mRNA levels contained protease-cleavable PEG and the appropriate siRNA.

Further evidence for proteolytic processing of PEG-FCit-PABC modified polymers was observed upon incubation of masked polymer in the presence of rat liver microsomal proteases. It is difficult to interpret EAB molecular weights by size exclusion retention times due to the amphipathic nature and cationic charge of the polymer, which cause retention time changes independent of molecular weight. For this reason, a reagent was prepared that linked the chromophore *p*-nitrophenylethylenediamine (PNPEDA) to EAB via an ACit-PABC bond (see supplemental section for description). The incubation of polymer modified with PNPEDA-ACit-PABC with rat lysosomal extract produced a small molecule chromophore with an approximate half-life of 1 h (see supplemental Fig. 6), which provides an estimate of the lability of ACit-PABC and is consistent with cleavage of citrulline-PABC amide bond by lysosomal proteases.

In addition to the FCit motif, a variety of peptide sequences composed of 1–2 residues with various charged and uncharged residues were tested (see Fig. 1 of supplementary data). The modification of the delivery polymer with a variety of dipeptide motifs allows for functional delivery. This limited data set is consistent with the cathepsin motif of a hydrophobic (F, V, A) residue followed by a polar residue (Cit, dimethyl-K, N) as good substrates for unmasking. The only naturally-occurring L amino acid-based modifications that appear to drastically reduce delivery are the modifications with only one amino acid, which suggests that single amino acid derivatives are relatively poor substrates for proteolysis.

Functional delivery using a mixture of protease-labile PEG reagents and pH-labile targeting reagents was a relatively facile demonstration of the potential of using protease substrates in delivery vehicles. In order to produce targeted delivery vehicles that rely solely on proteases for unmasking and delivery, PBAC reagents containing a hepatocyte-targeting NAG ligand were synthesized (Fig. 4). Initially, NAG-containing reagents using the FCit dipeptide motif were synthesized; however, formulations of polymers modified with NAG-FCit had limited solubility and were prone to gel or form viscous solutions. To circumvent this propensity, NAG-containing reagents using the more water-soluble dipeptide alanine-citrulline (ACit) motif were synthesized (Fig. 4, NAG-ACit-PABC). To test the stereospecificity of peptide cleavage, reagents composed of non-natural D enantiomers of phenylalanine, alanine and citrulline were also synthesized (fcit and acit).

siRNA delivery DPCs composed of polyacrylate EAB modified with PEG-FCit and NAG-ACit were formulated as before where PEG reagents were screened except the putative protease cleavable NAG reagent NAG-ACit-PABC was used instead of pH-labile CDM-NAG. One formulation (non-targeted group in Fig. 7) was not treated with NAG reagent, but was extensively modified with a second addition of PEG-FCit-PABC.

Formulations of polymer-conjugated FVII siRNA, which were modified with protease cleavable PEG and NAG were injected into mice via tail vein (n = 5, 20 μg of siRNA conjugated to 200 μg of polymer in 200 μL volume). Serum and liver tissue samples were collected 7 days after injection and assayed for fVII enzyme activity and FVII mRNA expression. The results presented in Fig. 7 demonstrate functional delivery of siRNA based solely on enzymatic unmasking of the membrane interactive polymer. Both FVII mRNA and enzyme levels were decreased 75% after a single injection of 0.25 mg/kg siRNA formulated with 2.5 mg/kg of masked polymer. Consistent with an enzymatic process, functional delivery is stereospecific as formulations modified with reagents containing D enantiomer amino acids were not functional in vivo.

No knockdown was observed in DPC formulations where only PEG masking reagents were used (non-targeted group in Fig. 7). The in vivo dispositions of NAG-targeted and PEG-only formulations were studied using radiolabeled polymer. The NAG-modified formulation accumulated rapidly in the liver, with 80% of dose associated with the liver 1 h postinjection. The PEG-only formulation had a relatively low (<10%) and constant amount associated with the liver (see supplemental Fig. 5). The PEG-only formulation circulates relatively long term with a half-life of circulation of 11 h (see Fig. 5a of supplemental). While the PEG-only formulation does not target any tissue selectively, the serum

Table 1
Physical characteristics of protease-sensitive delivery vehicles.

Attribute	Value
Molecular weight of EAB	50,000
Polymer charge density (MW/charge)	150/+
NAG ligands per vehicle	250
siRNA:polymer (mol:mol)[a]	1:3
Size	20 nm
ζ-potential	−10 mV
PEG:NAG ratio (mol:mol)	1:3
siRNA conjugation efficiency	90%
Molecular weight of vehicle (polymer + siRNA + masking groups)	225,000

[a] Calculations for 10:1 weight ratio polymer to siRNA formulated vehicle.

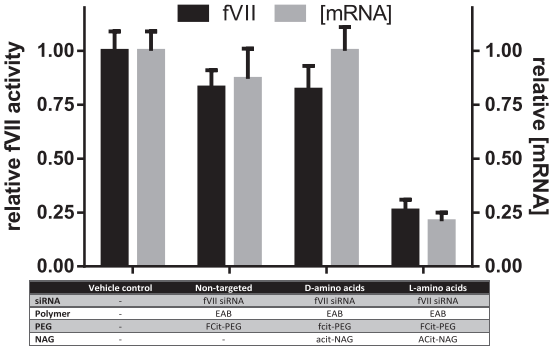


Fig. 7. fVII activity (left axis, black bars) and mRNA quantitation (right axis, gray bars) after intravenous administration of DPC formulations modified solely with protease-labile modifications. Formulations were injected via tail vein in mice (n = 4). Samples were taken 7 days postinjection. fVII activities and mRNA levels were normalized to mice treated with isotonic glucose.

circulation time suggests that the vehicles are stable and relatively non-interactive with components such as the extracellular matrix or cellular membranes that would prevent circulation.

The liver-associated material for targeted and untargeted formulations was evaluated using confocal microscopy. As can be seen from Fig. 8A, the NAG-modified formulation accumulates preferentially in hepatocytes (cells containing larger nuclei). While not quantitative, the PEG-modified material was less intense and not hepatocyte-specific (Fig. 8B).

To determine the efficacy of a 10:1 polymer:siRNA weight ratio formulation, a dose titration study was performed and is presented in Fig. 9. The dose required to reduce FVII enzymatic activity by 50% relative to untreated animals is roughly 1.25 mg/kg with respect to polymer and 0.125 with respect to siRNA.

In the studies described in Figs. 6–7 and 9, the polymer to siRNA ratio was held at 10:1 ratio, but different ratios were used. To test whether siRNA or polymer was limiting at the ED_{50} observed in Fig. 9 (1.25 mg/kg polymer with 0.125 mg/kg siRNA), a study was performed where the dose of polymer was held constant at 1.25 mg/kg and the siRNA dose was increased to 0.25 mg/kg. This increase in siRNA dose changed the molar ratio from 3:1 to 1.5:1 polymer to siRNA. No further increase in knockdown was observed at this increased siRNA dose, which suggests that the ED_{50} of 1.25 mg/kg polymer at 10 to 1 polymer:siRNA ratio is due to a lack of delivery polymer and not siRNA. See Fig. 3 of supplementary data section for dose titration of siRNA at constant 1.25 mg/kg polymer dose.

The presumed mechanism of endosomal lysis, which leads to cytoplasmic access of the siRNA, relies upon increased polymer–membrane interactions as masking groups are removed and the neutral polymer becomes positively charged. The observation that the ED_{50} with respect to polymer is equivalent at 3 and 1.5 to 1 polymer to siRNA molar ratios suggests that the mechanism of delivery requires multiple polymers (or interactions between polymers) to affect cytoplasmic delivery of siRNA, and that multiple polymers may need to be in the same intracellular compartment in order for the polymers to increase membrane permeability.

Maximum knockdown was observed 7 days after injection (A time course of fVII activity is presented in Fig. 2 of supplementary data.). The fact that mRNA reduction and presumably access to the cytoplasm require days during which the siRNA is exposed to nucleases suggests that the siRNA is protected from nucleases during the time between injection and its cytoplasmic entry and incorporation into the RNA-induced silencing complex. The research presented in this report does not address the source of siRNA stability, but some of the protection of the oligonucleotide undoubtedly comes from the 2′ modifications

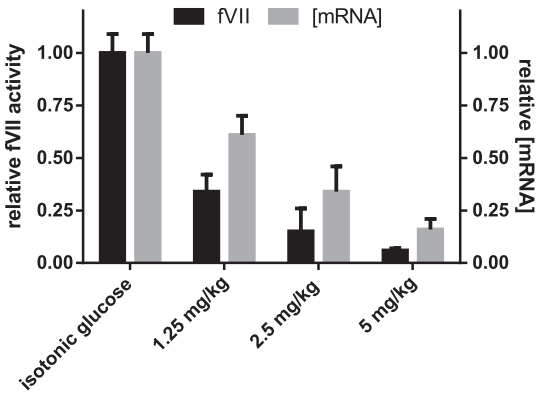


Fig. 9. fVII activity (left axis, black bars) and mRNA quantitation (right axis, gray bars) after intravenous administration of DPC formulations of various siRNA and polymer doses using a fixed siRNA:polymer weight ratio of 10:1. Formulations were injected via tail vein in mice ($n = 4$). Samples were taken 7 days postinjection. fVII activities and mRNA levels were normalized to mice treated with isotonic glucose.

[28], while further nuclease resistance may be derived from covalent attachment to masked polymer, which is becoming positively charged and hence nuclease protective upon proteolysis in the endosomes/lysosomes.

For all of the masking reagents tested, the resulting formulations are small (20 nm) and have a negative surface charge (−10 mV ζ-potential) due to the presence of the anionic siRNA (Table 1). In addition, the delivery vehicles are stable and nonaggregating, which enables purification and long-term storage. These attributes suggest that these formulations would be amenable to subcutaneous injections, which require stability in the lymphatic system before accessing the vasculature. Indeed, injection of FCit-PABC modified EAB-siRNA conjugates (5 mg/kg EAB and 0.5 mg/kg siRNA) results in greater than 80% knockdown of FVII 7 days after subcutaneous injection in mice (data not shown).

A comparison of subcutaneously and intravenously administered protease-labile DPCs in primates is presented in Fig. 10. To minimize any potential toxicity from *p*-nitrophenol (PNP), which is generated upon reaction of polymer amine with PNP-activated carbonate, the formulations were purified by ultrafiltration to remove small molecules such as PNP and any unconjugated PEG or NAG reagents. In order to decrease the volume of injection, the purified formulations were lyophilized and reconstituted in isotonic glucose at 200 mg/mL of polymer (20 mg/mL siRNA). The purified, concentrated formulations were injected at 5 mg/kg EAB polymer formulated with 0.5 mg/kg siRNA against primate fVII either intravenously or subcutaneously into

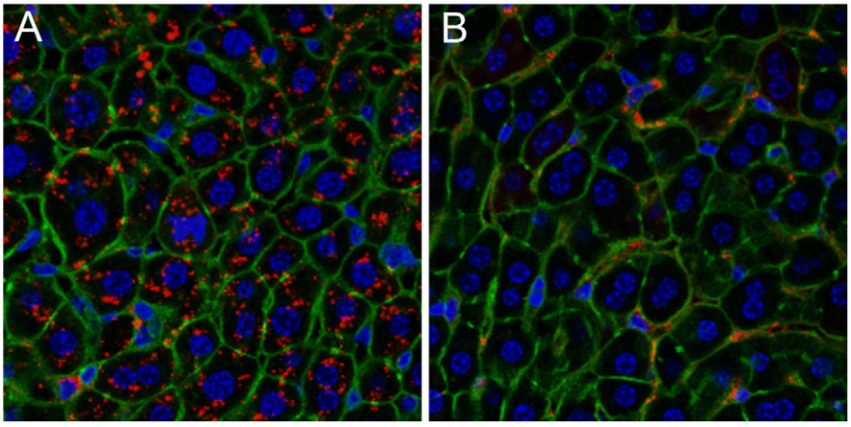


Fig. 8. Confocal micrographs of Cy3-labeled EAB in mouse liver sections. Confocal images of Cy3-labeled EAB modified with NAG/PEG (A) or PEG alone (B) using PABC reagents. Livers were harvested, fixed, and counterstained with To-Pro-3 to visualize nuclei (blue) and Alexa-633 phalloidin to visualize cell outlines (green). Each image comprised a flattened projection of 11 optical images (0.4 mm each) to represent combined fluorescence signals from a 4-mm-thick section.

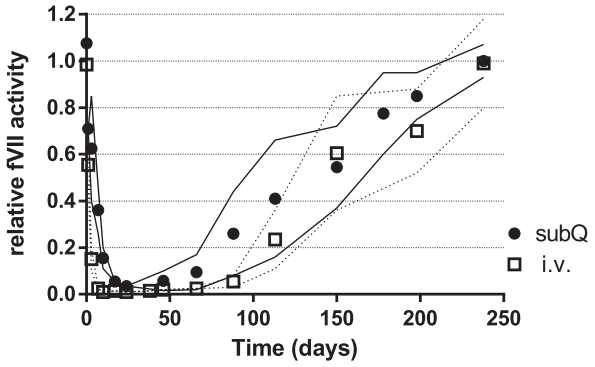


Fig. 10. fVII activity after intravenous or subcutaneous administration of DPCs at 5 mg/kg polymer in non-human primates (N = 2). Serum fVII activities were normalized to preinjection levels in non-human primate after intravenous and subcutaneous administrations. Lines represent individual animals and symbols (squares and circles) represent the average of both animals at each time point. Solid lines and filled circles are data for subcutaneous administration. Dashed lines and open squares are data for intravenous administration of DPC formulation.

cynomolgus monkeys (NHP, n = 2). At various time points after injection, plasma samples were collected. For either routes of administration, a 99% reduction of FVII was observed.

Maximal knockdown in primates was measured 10 days postinjection for intravenous injection and 24 days for subcutaneous injection. The difference in knockdown kinetics between the routes of administration suggests that the subcutaneously injected formulation is delayed in the lymphatic system before arrival in the liver. A prolonged duration of knockdown was observed with measurable reduction in FVII activity up to 200 days after a single injection of siRNA. The prothrombin time (PT), which is the amount of time for plasma to clot after the addition of tissue factor, was also measured. Blood clotting kinetics are a function of FVII levels, and an expected phenotypic increase in PT was observed as fVII levels decreased (see Fig. 4 of supplementary data).

4. Conclusion

A new class of siRNA delivery vehicles that are dependent upon intracellular protease activity has been developed. These delivery vehicles are based upon a general strategy where membrane interactive polymers are reversibly masked to inhibit membrane interactions. The key to functional delivery is control of the unmasking reaction such that it occurs selectively after internalization. The masking chemistry described is reversed by proteolysis of peptide linkages between hydrophilic groups and membrane interactive polymers. The hydrophilic groups may be PEG, which prevent nonspecific interactions, and/or cell-specific targeting groups such as NAG, which target the formulation to hepatocytes. Consistent with the enzymatic control of delivery, the unmasking is substrate specific as reagents containing non-natural D enantiomer amino acids do not allow for functional delivery of siRNA in vivo.

While observations such as the attenuation of hemolysis after PEG modification (Fig. 2b) and the dependence of functional siRNA delivery on modification via L amino acids (Fig. 7) are consistent with designed mechanism of delivery, the exact intracellular events such as the extent of unmasking required for delivery and the extent of the endo/lysosomal membrane disruption whereby the modified polymers provide access of the siRNA to cytoplasm are outside the scope of the work presented. Presumably, EAB after extensive proteolytic removal of hydrophilic PEG and NAG produces — like other amphipathic polycations [17] — nanometer-sized holes in membranes, which causes influx of small molecules and ultimately membrane disruption via a colloid-osmotic mechanism of lysis.

The protease-masked delivery vehicles are small and non-aggregating. The stability of the DPC masking chemistry in the absence of proteases ensures that the vehicles stay intact and not positively-charged until they reach their intended intracellular target. In addition, the covalent attachment of siRNA and polymer prevents displacement of siRNA from the delivery polymer. These attributes enable the transit of the siRNA and its DPC delivery vehicle in the lymphatic and vasculature systems before reaching the target liver tissue.

In addition to the stability required for functional delivery after subcutaneous delivery, there are no observed changes in physical properties (Table 1) or in vivo efficacy after storage for at least a year as either a lyophilized solid or in solution, which will aid in the manufacturing and analytics of therapeutics based upon protease masking.

The liver, due to its unique vasculature structure, is relatively easy to access. In order to deliver to relatively less accessible tissues such as tumors, formulations must be small and long-circulating in order for extravasation to occur. The in vitro stability and the in vivo stability required for subcutaneous administration of protease-dependent DPCs are consistent with the requirements needed for extrahepatic targeting where extravasation is not as facile as for the liver. Tissue-selective targeting also requires ligand-mediated delivery. Functional delivery of siRNA by DPCs to hepatocytes is dependent on the NAG targeting ligand, which suggests that protease sensitive DPCs can — with the attachment of appropriate targeting ligands — be actively targeted to other tissues. For these reasons (size, stability and targetability), protease-sensitive delivery vehicles are a potentially attractive strategy for extrahepatic delivery.

Acknowledgments

The authors acknowledge Alex Sokoloff for the preparation of rat liver lysosomes for digestion assay, Guofeng Zhang for tissue processing and Lauren Leckwee for confocal imaging of fluorophore-labeled formulations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.04.012.

References

[1] V. Knorr, L. Allmendinger, G.F. Walker, F.F. Paintner, E. Wagner, An acetal-based PEGylation reagent for pH-sensitive shielding of DNA polyplexes, Bioconjug. Chem. 18 (2007) 1218–1225.
[2] E.R. Gillies, A.P. Goodwin, J.M. Frechet, Acetals as pH-sensitive linkages for drug delivery, Bioconjug. Chem. 15 (2004) 1254–1263.
[3] N. Murthy, J. Campbell, N. Fausto, A.S. Hoffman, P.S. Stayton, Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides, J. Control. Release 89 (2003) 365–374.
[4] G.F. Walker, C. Fella, J. Pelisek, J. Fahrmeir, S. Boeckle, M. Ogris, E. Wagner, Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo, Mol. Ther. 11 (2005) 418–425.
[5] H. Bruyere, A.D. Westwell, A.T. Jones, Tuning the pH sensitivities of orthoester based compounds for drug delivery applications by simple chemical modification, Bioorg. Med. Chem. Lett. 20 (2010) 2200–2203.
[6] C. Masson, M. Garinot, N. Mignet, B. Wetzer, P. Mailhe, D. Scherman, M. Bessodes, pH-sensitive PEG lipids containing orthoester linkers: new potential tools for nonviral gene delivery, J. Control. Release 99 (2004) 423–434.
[7] X. Guo, F.C. Szoka Jr., Steric stabilization of fusogenic liposomes by a low-pH sensitive PEG–diortho ester–lipid conjugate, Bioconjug. Chem. 12 (2001) 291–300.
[8] J. Heller, J. Barr, S.Y. Ng, K.S. Abdellauoi, R. Gurny, Poly(ortho esters): synthesis, characterization, properties and uses, Adv. Drug Deliv. Rev. 54 (2002) 1015–1039.
[9] J.A. Boomer, M.M. Qualls, H.D. Inerowicz, R.H. Haynes, V.S. Patri, J.M. Kim, D.H. Thompson, Cytoplasmic delivery of liposomal contents mediated by an acid-labile cholesterol-vinyl ether-PEG conjugate, Bioconjug. Chem. 20 (2009) 47–59.
[10] B.R. Lee, K.T. Oh, Y.T. Oh, H.J. Baik, S.Y. Park, Y.S. Youn, E.S. Lee, A novel pH-responsive polysaccharidic ionic complex for proapoptotic D-(KLAKLAK)2 peptide delivery, Chem. Commun. (Camb.) 47 (2011) 3852–3854.
[11] M. Meyer, A. Zintchenko, M. Ogris, E. Wagner, A dimethylmaleic acid-melittin-polylysine conjugate with reduced toxicity, pH-triggered endosomolytic activity and enhanced gene transfer potential, J. Gene Med. 9 (2007) 797–805.
[12] D.B. Rozema, K. Ekena, D.L. Lewis, A.G. Loomis, J.A. Wolff, Endosomolysis by masking of a membrane-active agent (EMMA) for cytoplasmic release of macromolecules, Bioconjug. Chem. 14 (2003) 51–57.

[13] K.Y. Choi, M. Swierczewska, S. Lee, X. Chen, Protease-activated drug development, Theranostics 2 (2012) 156–178.
[14] D.B. Rozema, D.L. Lewis, D.H. Wakefield, S.C. Wong, J.J. Klein, P.L. Roesch, S.L. Bertin, T.W. Reppen, Q. Chu, A.V. Blokhin, J.E. Hagstrom, J.A. Wolff, Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 12982–12987.
[15] D. Wakefield, D. Rozema, N.A. Rossi, L. Almeida, A. Perillo-Nicholas, Poly(acrylate) Polymers for In Vivo Nucleic Acid Delivery, USPTO, US, 2012. (2013/0317079 A1).
[16] C. Barner-Kowollik, G. Moad, The mechanism and kinetics of the RAFT process: overview, rates, stabilities, side reactions, product spectrum and outstanding challenges, in: C. Barner-Kowollik (Ed.), Handbook of RAFT Polymerization, Wiley, 2008.
[17] I. Sovadinova, E.F. Palermo, R. Huang, L.M. Thoma, K. Kuroda, Mechanism of polymer-induced hemolysis: nanosized pore formation and osmotic lysis, Biomacromolecules 12 (2011) 260–268.
[18] K. Kuroda, G.A. Caputo, W.F. DeGrado, The role of hydrophobicity in the antimicrobial and hemolytic activities of polymethacrylate derivatives, Chemistry 15 (2009) 1123–1133.
[19] P.L. Carl, P.K. Chakravarty, J.A. Katzenellenbogen, A novel connector linkage applicable in prodrug design, J. Med. Chem. 24 (1981) 479–480.
[20] G.M. Dubowchik, R.A. Firestone, Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin, Bioorg. Med. Chem. Lett. 8 (1998) 3341–3346.
[21] G.M. Dubowchik, R.A. Firestone, L. Padilla, D. Willner, S.J. Hofstead, K. Mosure, J.O. Knipe, S.J. Lasch, P.A. Trail, Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific in vitro anticancer activity, Bioconjug. Chem. 13 (2002) 855–869.
[22] M.S. Sutherland, R.J. Sanderson, K.A. Gordon, J. Andreyka, C.G. Cerveny, C. Yu, T.S. Lewis, D.L. Meyer, R.F. Zabinski, S.O. Doronina, P.D. Senter, C.L. Law, A.F. Wahl, Lysosomal trafficking and cysteine protease metabolism confer target-specific cytotoxicity by peptide-linked anti-CD30-auristatin conjugates, J. Biol. Chem. 281 (2006) 10540–10547.
[23] Y. Yang, V.H. Thomas, S. Man, K.G. Rice, Tissue targeting of multivalent GalNAc Le(x) terminated N-glycans in mice, Glycobiology 10 (2000) 1341–1345.
[24] R. Gref, M. Luck, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk, R.H. Muller, 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption, Colloids Surf. B: Biointerfaces 18 (2000) 301–313.
[25] A.D. Judge, G. Bola, A.C. Lee, I. Maclachlan, Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo, Mol. Ther. 13 (2006) 494–505.
[26] M. Sioud, G. Furset, L. Cekaite, Suppression of immunostimulatory siRNA-driven innate immune activation by 2′-modified RNAs, Biochem. Biophys. Res. Commun. 361 (2007) 122–126.
[27] S.C. Wong, J.J. Klein, H.L. Hamilton, Q. Chu, C.L. Frey, V.S. Trubetskoy, J. Hegge, D. Wakefield, D.B. Rozema, D.L. Lewis, Co-injection of a targeted, reversibly masked endosomolytic polymer dramatically improves the efficacy of cholesterol-conjugated small interfering RNAs in vivo, Nucleic acid Ther. 22 (2012) 380–390.
[28] J.B. Bramsen, J. Kjems, Development of therapeutic-grade small interfering RNAs by chemical engineering, Front. Genet. 3 (2012) 154.