Bioconjugate Chemistry

Article

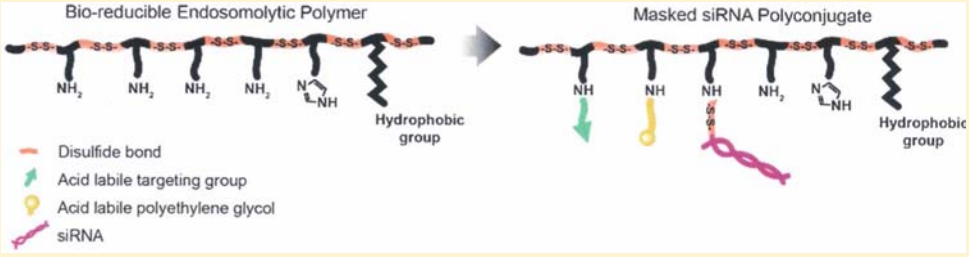
pubs.acs.org/bc

Endosomolytic Bioreducible Poly(amido amine disulfide) Polymer Conjugates for the *in Vivo* Systemic Delivery of siRNA Therapeutics

Rubina Giare Parmar,*[,†] Marina Busuek,[†] Eileen S. Walsh,[‡] Karen R. Leander,[‡] Bonnie J. Howell,[‡] Laura Sepp-Lorenzino,[‡] Eric Kemp,[§] Louis S. Crocker,[§] Anthony Leone,[§] Christopher J. Kochansky,[∥] Brian A. Carr,[∥] Robert M. Garbaccio,[†] Steven L. Colletti,[†] and Weimin Wang[†]

[†]Department of Medicinal Chemistry, [‡]Department of RNA Biology, [§]Department of Pharmaceutical Sciences, and [∥]Department of Pharmacokinetic Pharmacodynamic Drug Metabolism, Merck & Co. Inc, West Point, Pennsylvania, United States

Supporting Information



ABSTRACT: Efficient siRNA delivery is dependent not only on the ability of the delivery vehicle to target a specific organ but also on its ability to enable siRNA entry into the cytoplasm of the target cells. Polymers with endosomolytic properties are increasingly being used as siRNA delivery vehicles due to their potential to facilitate endosomal escape and intracellular delivery. Addition of disulfide bonds in the backbone of these polymers was expected to provide degradability through reduction by glutathione in cytosol. This paper describes the synthesis of new endosomolytic bioreducible poly(amido amine disulfide) polymers whose lytic potential can be masked at physiological pH, but can be restored at acidic endosomal pH. These polymer conjugates gave good *in vitro* knockdown (KD) and did not demonstrate cytotoxicity in a MTS assay. Efficient mRNA KD for apolipoprotein B in mouse liver was observed with these polyconjugates following intravenous dosing.

INTRODUCTION

Since the discovery of RNA interference (RNAi), an evolutionarily conserved pathway for post-transcriptional gene silencing,[1] and the demonstration of synthetic short interfering RNA (siRNA) to effectuate RNAi in mammalian cells,[2] siRNA-based therapeutics have attracted much attention as an emerging modality for the treatment of human diseases. The attractive features of siRNA include specificity, potency, duration, and the ability to target virtually all disease-relevant targets at the gene level including those which are non-targetable by conventional small molecules or monoclonal antibodies. Despite the recent progress in preclinical and clinical development,[3−5] the effective and safe delivery of siRNA to the disease site is still a major challenge. The current obstacles to siRNA delivery include enzymatic degradation of the oligonucleotide, rapid clearance, cellular uptake, endosomal escape, and tolerability of the delivery vehicle after systemic administration.[6]

Various polymeric systems have been developed as siRNA carriers to address these delivery issues.[7] Most of these systems form stable polyelectrolyte complexes (polyplex) with siRNA via electrostatic interactions between positively charged polymers and negatively charged oligonucleotide. However, concerns regarding the systemic toxicities caused by cationic amines,[8] poor endosomal escape, and premature separation of siRNA in biological matrices[9] have limited the polyplex approach from achieving practical application. New strategies aimed to address these shortcomings have recently been proposed.[10−12] In particular, the dynamic polymer conjugates reported by Rozema et al. rely on an amphiphilic lytic polymer poly(butyl and amino vinyl) ethers (PBAVE) to effect potent mRNA knockdown *in vivo*. This design makes use of acid-labile masking of the cationic amines to hide the positive charges and membrane lytic potential of the polymer and thereby reducing systemic toxicity. The masking agents e.g., poly(ethylene glycol) (PEG) and targeting ligand are expected to be cleaved from the polymer in the acidic endosome restoring the membrane lytic activity of the polymer for enhancing endosomal escape. The siRNA is covalently conjugated to the polymers via reducible disulfide bonds for better extracellular stability to avoid premature release of siRNA. The ability to target these polymer siRNA conjugates specifically to hepatocytes *in vivo* through N-acetyl-galactosamine ASGPR binding has also been demonstrated.[11] Although these polymer conjugate systems (polyconjugates) have demonstrated great potential for the systemic delivery of siRNA, tolerability is still a

Received: November 10, 2012
Revised: February 2, 2013
Published: March 18, 2013

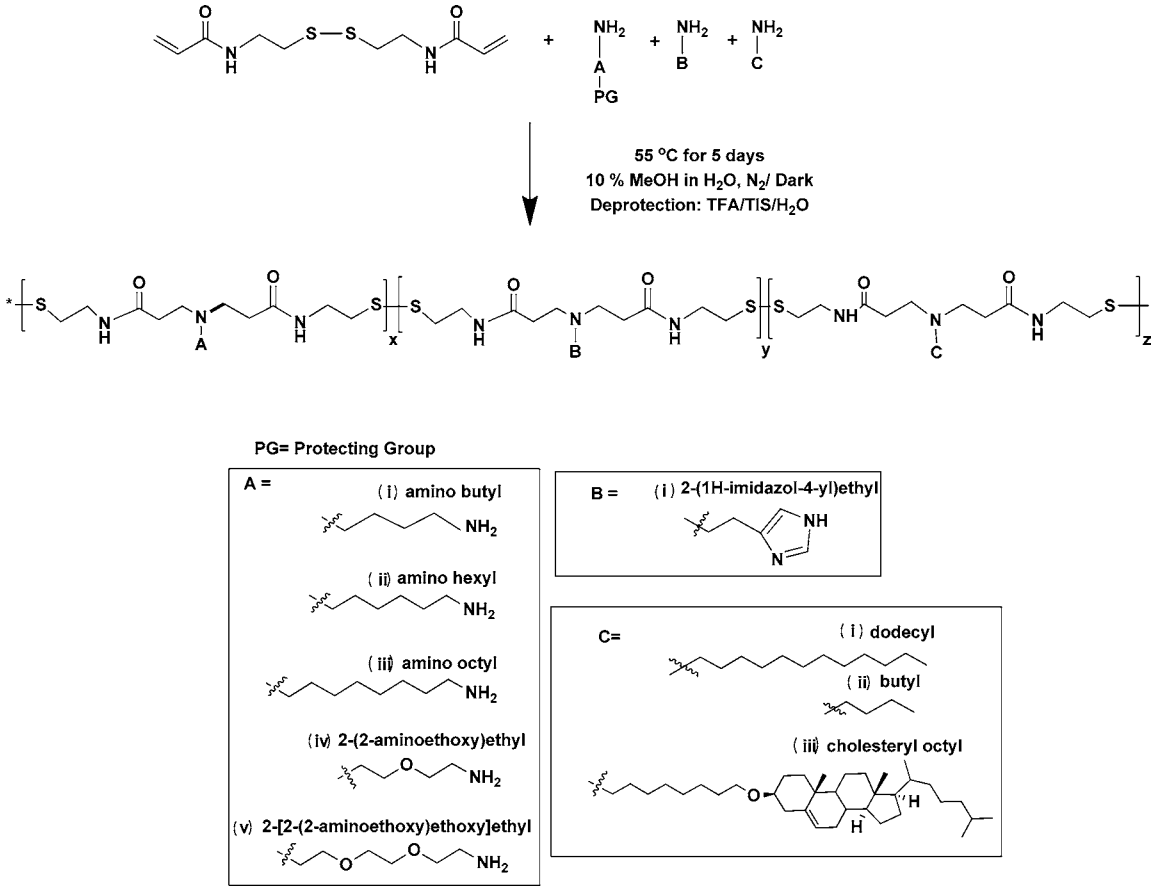


Figure 1. Chemical composition of polymers.

major concern due to the nondegradable nature of the vinyl ethyl polymer.

Bioreducible poly(amido amine) polymers containing disulfide linkages in the backbone have been reported to be efficient gene delivery carriers with much reduced toxicity compared to the conventional cationic polymers such as poly(ethyleneimine) (PEI) and poly(lysine).[13−15] The difference in glutathione (GSH) concentration between the extracellular milieu and intracellular environment (~1000× or 100×) suggests the potential for sufficient stability in circulation with rapid degradation of these polymers in the cytosol.[16] However, the studies on these polymers as siRNA delivery carriers have relied on the buffering capacity of the tertiary amines on the polymer backbone for endosomal escape and ionic polyplex for siRNA encapsulation. In this paper we have employed the key features of the polymer conjugate strategy along with this unique disulfide containing polymer to develop a new biodegradeable, pH responsive endosomolytic polymer conjugate for the delivery of therapeutic siRNA.

■ EXPERIMENTAL PROCEDURES

Materials. Cystaminebisacrylamide, *N*-(*tert*-butoxycarbonyl)-1,4-butanediamine, *N*-(*tert*-butoxycarbonyl)-1,6-hexanediamine, *N*-(*tert*-butoxycarbonyl)-1,8-octanediamine, butylamine, hexylamine, dodecylamine, trifluoroacetic acid (TFA), and triisopropylsilane (TIS) were purchased from Sigma.

Carboxydimethylmaleic anhydride-poly(ethylene glycol) (CDM-PEG; MW = 680 g/mol) and carboxydimethylmaleic anhydride-*N*-acetylgalactosamine (CDM-GalNAc) were synthesized according to the literature procedure.[11] The active siRNA targeting Apolipoprotein B[17] was used in the *in vitro* and *in vivo* experiments. The siRNA with an irrelevant sequence (Low Hex 9) was used as control (sequence shown below).

5′-amil-iB-*CU***AG***CU***GGA***CA***CG***UC***GA***UAT***sT**-iB-3′

3′-UsUGA*UC*GA*CCU*G*U*G*C*AG*C*UAU-5′

amil - amino linker; iB - Inverted deoxy abasic; *CU* - 2′-Fluoro (F); **AGT** - 2′-Deoxy; UGA - 2′-Methoxy (OMe); AU - Ribose; s - phosphorothioate linkage

General Procedure for the Synthesis of Poly(amido amine disulfide). Polymers. In a typical experiment (Figure 1), cystaminebisacrylamide (125 mg, 0.48 mmol, 1 equiv), *tert*-butyl [2-(2-aminoethoxy)ethyl]carbamate (boc-amine) (39 mg, 0.192 mmol, 0.4 equiv), histamine (16 mg, 0.144 mmol, 0.3 equiv), and dodecylamine (27 mg, 0.144 mmol, 0.3 equiv) were weighed in the reaction flask and 1 mL of 10% methanol solution in water was added to it. The reaction mixture was stirred at 55 °C for 5 days in the dark under nitrogen atmosphere. After 5 days, polymerization was quenched by adding 10 mol % excess of dodecylamine (9 mg, 0.048 mmol) to consume any unreacted bisacrylamide. Polymer was then precipitated with 100 mL diethyl ether and dried.

Deprotection of boc-amine was carried out by dissolving protected polymer in TFA/TIS/H_2O 95/2.5/2.5 solution for 30 min at room temperature. The crude polymer was precipitated from 100 mL diethyl ether and dried. It was further purified by dialysis using a 2k cutoff membrane against Milli-Q water and then lyophilized.

Polymer Characterization. ^{1}H NMR spectra were recorded on Varian spectrometer operating at 500 MHz. All NMR spectra were taken in deuterated methanol. ^{1}H NMR spectra

dx.doi.org/10.1021/bc300600a | *Bioconjugate Chem.* 2013, 24, 640−647

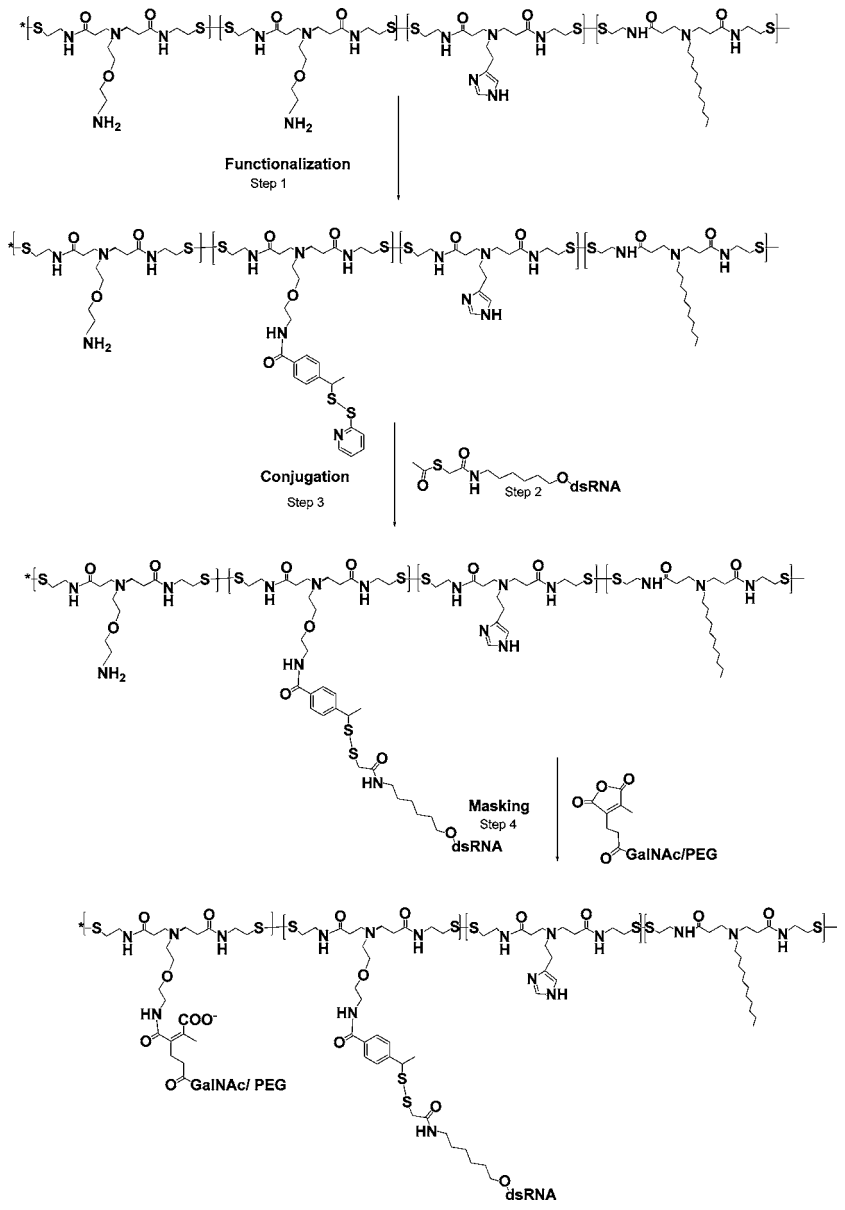


Figure 2. Polyconjugate Synthesis.

were in full accordance with the expected structures. No signals were present in the region between 5 and 7 ppm, indicating that these polymers do not have terminal acrylamides suggesting that polymer ends are amine capped. The molecular weight and polydispersity of the synthesized polymers were determined by gel permeation chromatography (GPC) relative to polystyrene standards (Sigma-Aldrich) using a Waters 2695, Waters 2414 RI detector, and a TOSOH TSK TSK-GEL Alpha-2500 column. Total amine content and the incorporation ratios of monomers in the polymers were calculated using NMR integration and by treating the polymers with excess of dithiothreitol (DTT) followed by liquid chromatography−mass spectrometry (LC-MS) analysis (see Supporting Information).

General Procedure for the Preparation of Polymer Conjugates. Poly(amidoamine disulfide) polymers were conjugated to siRNA and modified with targeting ligands (CDM-GalNAc) and poly(ethylene glycol) (CDM-PEG) using the following steps (Figure 2). Polymer to siRNA weight ratio was kept at 4:1.

Step 1: Activation of Polymer. About 26 mg (0.026 umol) of polymer was dissolved in ∼870 μL of 5 mM 3-{1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl}amino]propane-1-sulfonic acid (TAPS), pH 9 buffer. To this solution was added 39 μL 4-succinimidyloxycarbonyl-methyl-[α]-(2- pyridyldithio) toluene (SMPT) solution (1 mg/100 μL in dimethyl sulfoxide) corresponding to 1.5 wt % with respect to the polymer weight.

Step 2: Activation of siRNA. Apo B siRNA duplex with a hexyl amino linker at the 5′ terminal of passenger strand (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 equiv) was dissolved in 0.78 mL DMSO. The SATA solution was added over 1 min and the clear, colorless reaction mixture was stirred at 0−5 °C for 2 h. After 2 h, the reaction mixture was sampled and analyzed by high performance liquid chromatography (HPLC) for completion of the conjugation. If >5% siRNA remains unreacted, another charge of SATA in

dx.doi.org/10.1021/bc300600a | *Bioconjugate Chem.* 2013, 24, 640−647

DMSO (33 mg, 2.0 equiv) was added and the reaction was aged at 0−5 °C for completion of the SATA conjugation (confirmation by HPLC). When there was <5% unreacted siRNA remaining indicated by HPLC, the reaction mixture was purified by tangential flow filtration (TFF) dialysis using water (~2 L) to remove any remaining SATA/succinimides. The recovered purified solution was lyophilized to a white fluffy solid. The recovery was typically around 95% and the purity was greater than 70% confirmed by HPLC.

Step 3: Polymer-siRNA Conjugation. The SMPT activated polymer (26 mg) was diluted with additional 5 mM TAPS 5% glucose buffer pH 9 (13 mL) resulting in a final polymer concentration of ~2 mg/mL. About 6.6 mg of SATA-siRNA was added to the activated polymer solution with stirring at room temperature. After stirring overnight the reaction mixture was used in the final masking step.

Step 4: Masking of Polyconjugates. To the polymer-siRNA conjugate was added 10 mg of TAPS free base. In a separate vial, CDM-PEG (40 ug, 0.065 umol, 2.5 equiv) and CDM-GalNAc (90 ug, 0.195 umol, and 7.5 equiv) were combined. The siRNA-polymer conjugate solution was then transferred into the vial containing CDM-PEG and CDM-GalNAc and the solution was stirred for 1 h at room temperature.

Red Blood Cell (RBC) Hemolysis Analysis. Human blood was collected in 10 mL EDTA Vacutainer tubes. A small aliquot was assessed for evidence of hemolysis by centrifugation at 15000 RCF for 2 min and nonhemolyzed samples were carried forward into the assay. Red blood cells (RBCs) were washed three times in either 150 mM sodium chloride (NaCl)/20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.4, or 150 mM NaCl/20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), pH 7.5 by centrifuging at 1700*g* for 3 min and resuspending in the same buffer to yield a 10× dilution. RBCs were then diluted in appropriate pH buffer to yield approximately 10^8 cells in suspension. A 10× stock concentration of each test agent was prepared and a 10 point, 2-fold dilution was performed in appropriate pH buffers. The diluted test agents were added to the RBCs in appropriate pH buffers in Costar 3797 round-bottom 96 well plates. Solutions were mixed 6 to 8 times and the micro titer plate was covered with a low evaporation lid and incubated in a 37 °C warm room or incubator for 30 min to induce hemolysis. The plate was then centrifuged at 1700*g* for 5 min and 150 μL supernatants were transferred to a Costar 3632 clear bottom 96 well plate. Hemoglobin absorbance was read at 541 nm using a Tecan Safire plate reader and percent hemolysis was calculated assuming 100% lysis to be measured by the hemoglobin released by RBCs in 1% Triton X-100.

In Vitro HepG2 Gene Silencing and Toxicity (bDNA/MTS Assay). HepG2 cells were plated in 96-well micro titer plates at 6000 cells/well and incubated overnight at 37 °C to allow cell adherence. A 10× stock of PCs (polyconjugates) were prepared in media and 20 μL 10× PC was added to 180 μL media already in wells resulting in 1× final treatment and a 300−0 nM 10-point half log titration, based on siRNA concentration. Cells were incubated with PCs in 37 °C CO_2 incubator for 24−72 h. MTS Toxicity Assay was performed on 24−72 h treated cells and cytotoxicity was assessed by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega #G3581, Madison, WI). 40 μL MTS Solution was added incubated in 37 °C CO_2 incubator 1 h; absorbance at 490 nm was read on Tecan Safire. Cells were then washed 3× in PBS and 150 μL/well bDNA DLM Lysis Buffer (Panomics "Quantigene" 1.0 bDNA kit #QG0002, Fremont, CA) was added. The plate was then incubated at 37 °C in a warm room for 30 min. Lysates were removed and frozen at −70 °C overnight. The next day, all cell lysates were thawed at RT and 20 μL of each lysate was removed and used for determination of total protein using a Micro BCA Protein Assay kit (Pierce #23235, through Thermo Scientific, Rockford, IL). Absorbance was measured on a Tecan Safire: Wavelength = 562nM, Plate = Costar96 ft, Number of Reads = 100, Time between Reads = 5. 50 μL of each lysate was also used to determine mRNA expression levels in cells treated with ApoB siRNA.

ApoB mRNA knockdown was determined using a Quantigene 1.0 bDNA Assay (Panomics #QG0002 Lot #51CW36, Fremont, CA), a kit designed to quantitate RNA using a set of target-specific oligonucleotide probes.

In Vivo Studies. CD1 mice (n = 4, each group) were tail vein injected with polyconjugates at a dose of 3 mg/kg in a volume of 0.2 mL, 10 mM TAPS 5% glucose buffer, pH 9, vehicle. 48 h post dose, mice 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 Cat. No. Mm01545156_m1). The PCR reaction was run on an ABI 7500 instrument with a 96-well Fast Block. The ApoB mRNA level is normalized to the housekeeping PPIB mRNA and GAPDH. PPIB and GAPDH mRNA levels were determined by RT-PCR using a commercial probe set (Applied Biosytems Cat. No. Mm00478295_m1 and Mm4352339E_m1). Results are expressed as a ratio of ApoB mRNA/PPIB/GAPDH mRNA. All mRNA data is expressed relative to the vehicle control.

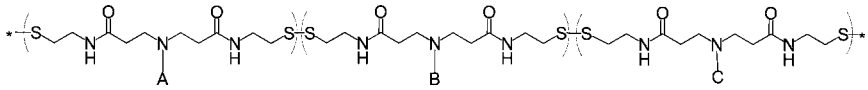
■ RESULTS AND DISCUSSION

Polymer−siRNA conjugates have shown promising potential for systemic siRNA delivery. The dynamic polyconjugate delivery system described by Rozema et al.[11] has demonstrated effective knockdown of two endogenous genes (apolipoprotein B and peroxisome proliferator activated receptor alpha) in mouse liver, and these polyconjugates were nontoxic based on serum liver enzymes and cytokine levels in treated mice. One potential liability of this polymer conjugate is the non-biodegradable nature of the cationic, lytic poly(vinyl ether) backbone in terms of excretion and chronic toxicity.

In this paper, we aim to employ features of this design and develop a polymer−siRNA conjugate that has the potential to degrade to low MW fragments after the delivery of siRNA to the target hepatocytes. This delivery system consists of an endosomolytic poly(amido amine disulfide) polymer, which has pendant amines that can be reversibly masked with acid sensitive maleic anhydride based targeting ligands and poly(ethylene glycol). The polymer has multiple disulfide linkages in the backbone that are expected to impart biodegradability in cytosol as a result of the higher intracellular concentration of glutathione. Our strategy relied on an *in vitro* screening funnel to identify polymers with the greatest potential for *in vivo* activity as polymer conjugates. As a first step, multiple amphiphilic poly(amido amine disulfide) polymers were synthesized and screened in an RBC hemolysis assay with the intent of optimizing lytic activity. The polymers active in the

dx.doi.org/10.1021/bc300600a | *Bioconjugate Chem.* 2013, 24, 640−647

Table 1. Polymerization Feed Ratio and Polymer Red Blood Cell Hemolysis Data



	A	B	C		RBC hemolysis data	
polymer #	amine monomer (molar ratio)	imidazole monomer (molar ratio)	lipophilic monomer (molar ratio)	Mn = kDa (PDI)	pH 7.4 (EC_{50}) mg/mL	pH 5.4 (EC_{50}) mg/mL
1	2-(2-aminoethoxy)ethyl (80)	0	dodecyl (20)	8.18 (1.9)	0.11	0.3
2	2-(2-aminoethoxy)ethyl (50)	0	dodecyl (50)	6.17 (1.6)	0.04	0.046
3	2-(2-aminoethoxy)ethyl (40)	2-(1*H*-imidazol-4-yl)ethyl (30)	dodecyl (30)	8.64 (3.2)	0.001	0.002
4	2-(2-aminoethoxy)ethyl (30)	2-(1*H*-imidazol-4-yl)ethyl (50)	dodecyl (20)	8.99 (3.5)	0.001	0.001
5	2-[2-(2-aminoethoxy)ethoxy] ethyl (70)	0	butyl (30)	10.67 (3.5)	>1	>1
6	2-[2-(2-aminoethoxy)ethoxy] ethyl (50)	0	butyl (50)	9.43 (3.0)	>1	>1
7	2-[2-(2-aminoethoxy)ethoxy] ethyl (50)	2-(1*H*-imidazol-4-yl)ethyl (10)	butyl (40)	9.86 (3.2)	>1	>1
8	2-[2-(2-aminoethoxy)ethoxy] ethyl (70)	0	dodecyl (30)	8.25 (2.6)	0.002	0.007

RBC lysis assay were conjugated with a siRNA targeting apolipoprotein B and masked with carboxydimethylmaleic anhydride poly(ethylene glycol) (CDM-PEG) and carboxydimethylmaleic anhydride-*N*-acetylgalactosamine (CDM-GalNAc) for *in vitro* knockdown (bDNA assay) and toxicity (MTS assay) screening. Based on *in vitro* knockdown data, polyconjugates were further selected for *in vivo* studies.

Synthesis, Characterization, and RBC Hemolysis Analysis of Polymers. The poly(amido amine disulfide) polymers were synthesized by the Michael addition[16] reaction between amines and bis-acrylamide. The stoichiometric ratio of amine to bisacrylamide was kept at 1:1 for all polymers. The advantage of this type of step-growth polymerization is the capability of modular synthesis to quickly generate polymer libraries with a variety of hydrophobic and hydrophilic moieties for structure activity relationship studies. The number average molecular weight (Mn) of the polymers was determined to be in the range from 6000 to 9000 with a polydispersity index of 2 to 3. The monomer incorporation ratio was determined by NMR integration and by polymer decomposition with 100 mM dithiothreitol (DTT) resulting in the formation of monomers that were analyzed using LC-MS (see Supporting Information). The observed ratio by both techniques was approximately the same as the feed ratio of monomers.

Polymers were screened in a red blood cell hemolysis assay to assess their membrane lytic potential. Hemoglobin absorbance was measured after incubating red blood cells with polymers at different pHs and plotted against polymer concentration. Hemolysis was determined at pHs 7.4 and 5.4 to simulate physiological and late endosomal pH. EC_{50} for hemolysis was determined for each polymer (see Table 1).

A series of polymers were synthesized using different molar ratios of amine (A), imidazole (B), and hydrophobic tail groups (C) as shown in Figure 1. These monomers were selected to make the polymer endosomolytic.

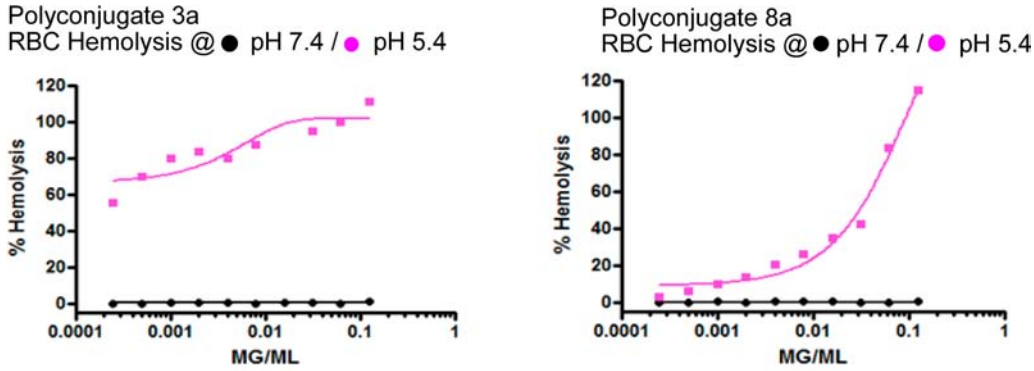
Pendant amine monomers (Figure 1A i–v) were selected based on their pK_a and masking efficiency with the CDM masking groups. Butylamine (A-i), hexyl amine (A-ii), and octyl amine (A-iii) have calculated pK_a values ranging from 10 to 10.7 and under standard formulation conditions[11] (pH 9 buffer) resulted in low masking efficiency. This poor reactivity may be due to amine protonation at the formulation pH. To resolve this problem, the less basic (2-(2-aminoethoxy)ethyl) amine monomer (A-iv) and 2-[2-(2-aminoethoxy)ethoxy]ethyl (A-v) (pK_a 8.75) were selected resulting in improved masking efficiency.

Monomers having a range of hydrophobic properties (Figure 1C i–iii) were copolymerized with the pendant amine monomers (Figure 1A i–v) to make the polymer amphiphilic and increase the lytic potential via enhancement of membrane interaction.[18] The RBC lysis assay demonstrated that increasing the incorporation of a hydrophobic monomer in the polymer increased the lytic activity. For example, an increase of the dodecyl content in polymer from 20% (polymer 1) to 50% (polymer 2), resulted in an increase in the lytic activity of the polymer from EC_{50} = 0.3 mg/mL to EC_{50} = 0.046 mg/mL at pH 5.4 (Table 1). However, increasing incorporation of the hydrophobic monomer in the polymer also decreased aqueous solubility. Polymers with more than 50% dodecyl monomer were not soluble in water (visual inspection) and could not be formulated for conjugation.

Two approaches were adopted to improve solubility while retaining lytic activity. The first approach was to incorporate histamine into the polymer backbone. The imidazole group of histamine, as reported in the literature,[19] may assist in endosomal escape through the proton sponge effect and in fusing the lipid bilayers through ionic interactions in a mildly acidic environment. Hemolysis results (Table 1) demonstrated the success of this approach. Polymer 3 and polymer 4 incorporating histamine have improved endosomolytic properties as compared to polymer 1 and polymer 2. An alternative approach to improve solubility was to incorporate a smaller butyl monomer instead of the dodecyl monomer (Polymers 5, 6, 7); however, lytic activity was not observed for up to 50% butyl monomer incorporation likely due to the reduced amphiphilic nature of these polymers. Attempts to incorporate cholesterol in the polymer resulted in poor solubility and low molecular weight polymers with broader polydispersity.

After screening, polymers 3, 4, and 8 were identified as having good lytic properties at endosomal pH (5.4) and were selected for *in vitro* bDNA assay along with polymer 1 as a negative control.

dx.doi.org/10.1021/bc300600a | *Bioconjugate Chem.* 2013, 24, 640–647



Polyconjugate #	Polymer #	EC_{50} @ 7.4	EC_{50} @ 5.4
Polyconjugate 3a	3	> 0.132	0.001
Polyconjugate 8a	8	> 0.15	0.06

Figure 3. Red blood cell hemolysis data of polyconjugates.

Preparation of Polyconjugates. Polymers (3, 4, and 8) showed good RBC lytic activity and were selected for further *in vitro* and *in vivo* evaluation along with a control polymer (1) of low lytic activity for comparison. All polymers were formulated with siRNA (both active and control) and CDMs as shown in Figure 2.

Polyconjugates (polymer conjugated with siRNA and masked with CDM) were prepared as previously described.[11] siRNA was conjugated to polymer through a disulfide bond due to its potential for siRNA release in the reductive environment of the cytosol. The polymers were first activated with SMPT followed by reaction with SATA-siRNA. The nitrogen/phosphorus (N/P) ratio was kept between 4 and 6, which corresponds to 4:1 polymer to siRNA weight ratio. The nitrogen/phosphorus (N/P) was calculated using both primary and tertiary amines of the polymer. Polymer−siRNA conjugates were then masked with CDM-GalNAc (7.5 equiv) and CDM-PEG (2.5 equiv) using 3:1 molar ratios, respectively.

The conjugation efficiency of siRNA to masked polymer was determined by SEC chromatography and was in general higher than 80% (see Supporting Information). The efficiency of the masking of amines by CDMs was determined to be 30−50% (see Supporting Information). Effort to further improve the masking efficiency of polyconjugates with CDM using higher molar equivalents of CDM's was not successful, presumably because of electrostatic repulsion and steric hindrance.

Reversible masking of the amines to reduce systemic toxicity under physiological conditions is a key feature of the design. pH responsive hydrolysis restores the membrane lytic activity in the acidic environment of the endosome. Thus, pH responsive behavior of these masked polyconjugates was also evaluated in the RBC lysis assay. Robust hemolysis was observed at pH 5.4 but not at pH 7.4 as shown in Figure 3, presumably due to the accelerated demasking of amines on the polymer at acidic pH.

***In Vitro* and *In Vivo* Data.** siRNA targeting apolipoprotein B (active siRNA) was used to assess the activity of these polyconjugates *in vitro* along with inactive Low Hex 9 siRNA sequence as a negative control. ApoB mRNA knockdown (KD) was determined in HepG2 cells using Quantigene 1.0 bDNA Assay which quantitates mRNA using a set of target-specific oligonucleotide probes. Cytotoxicity was evaluated using an MTS assay. As shown in Table 2, single digit nanomolar mRNA KD activity was observed with a 20- to 30-fold window to cytotoxicity *in vitro* with polyconjugates 3 and 8 of the active siRNA 3a and 8a, whereas no KD was observed for polyconjugates of the negative control siRNA 3b and 8b, demonstrating specific activity of the active siRNA. Interestingly, polyconjugate 1 with active siRNA (1a) showed no KD compared to polyconjugates 3 and 8, possibly due to the low lytic activity of polymer 1 (EC_{50} = 0.3 mg/mL @ pH 5.4).

Table 2. *In Vitro* (bDNA/MTS) Data of Polyconjugates with Active and Control siRNA

polyconjugate #	polymer #	siRNA active/ control	N/P ratio	bDNA IC_{50}(nM)	MTS IC_{50}(nM)
1a	1	Active	6	>300	>300
1b	1	Control	6	>300	>300
3a	3	Active	5	8	300
3b	3	Control	5	>300	>300
4a	4	Active	4	43	>300
4b	4	Control	4	>300	>300
8a	8	Active	6	6	163
8b	8	Control	6	>300	>300

Polyconjugate 4 shows a significantly lower *in vitro* knockdown efficiency than polyconjugate 3 (Table 2) even though the hemolysis capabilities of corresponding polymers (Polymers 3 and 4) are almost identical (Table 1). This may be due to differences in cellular uptake. Polymer of polyconjugate 4 has lower primary amine density (ratio of amine/imidazole/lipophilic group 30:50:20) as compared to the polymer of polyconjugate 3 (ratio of amine/imidazole/lipophilic group 40:30:30). The lower amine density leads to lower CDM-NAG targeting, which is required for hepatocyte uptake. Lower cellular uptake is expected to provide lower knockdown; however, this remains to be verified by changing the ratios of CDM-NAG. It is also possible that the difference in lipophilicity plays a role in the difference in knockdown observed. Also, the rate of demasking of CDM for polyconjugates 3 and 4 could be different, especially

dx.doi.org/10.1021/bc300600a | *Bioconjugate Chem.* 2013, 24, 640−647

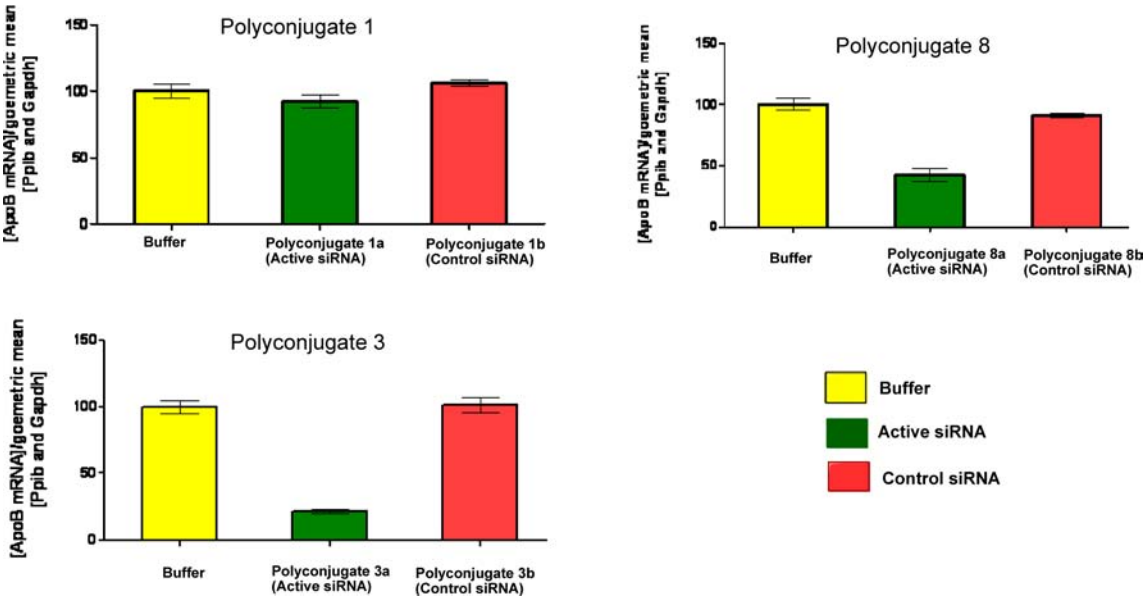


Figure 4. Liver Apo B mRNA expression (Mouse) at 3 mg/kg (48 h).

recognizing the difference in imidazole contents. The imidazole group has pK_a of 5.4, which may affect endosome acidification owing to its buffering capacity, thus affecting the rate of demasking.

Based on this *in vitro* data, polyconjugates 3a/3b and 8a/8b were selected for *in vivo* studies. Polyconjugates 1a/1b were included to test the translation of the bDNA assay to an *in vivo* experiment.

For *in vivo* studies, CD1 mice (n = 4, per group) were dosed with polyconjugates containing both active and control siRNA via bolus tail vein injection at 3 mg/kg and were sacrificed 48 h post dose. As shown in Figure 4, no ApoB mRNA knockdown was observed with polyconjugate 1a compared to its corresponding control polyconjugate 1b. In contrast, polyconjugates 3a and 8a showed robust gene silencing activity in mice; whereas no knockdown was observed with the corresponding Low Hex 9 negative controls (polyconjugates 3b and 8b).

Polyconjugates 3a and 8a with single-digit nanomolar mRNA knockdown in bDNA assay and with good lytic activity showed 80% and 60% knockdown, respectively, *in vivo*. Polyconjugate 3 and 8 have similar *in vitro* KD, but polyconjugate 3 has better *in vivo* activity as compared to polyconjugate 8 which may be due to different *in vivo* behavior (stability, binding, etc.) that was not approximated by an *in vitro* assay.

The knockdown observed with these polyconjugates approaches the activity observed with the PBAVE based system. In particular, polymer conjugate 3a showed 80% mRNA KD and suggests the important role of histamine incorporation in this design.

Biodegradability. Previous studies by Kim et al.[16] have shown that bioreducible poly(amido amine) polymers with disulfide linkages are stable under physiological conditions and degrade rapidly in the presence of glutathione (GSH). To establish the potential for biodegradation of the above-mentioned disulfide polymers in the cytosol, these disulfide-containing polymers were incubated in 5 mM GSH for 2 h. Monitoring of this reaction using reverse phase high pressure liquid chromatography (RP-HPLC) showed degradation of the polymer with GSH after 2 h.

Based on previous reports on the biodegradability of poly(amido amine disulfide) polymer, a new characterization technique was developed by treating the polymer with DTT (surrogate of GSH). Rapid degradation of the polymer backbone to the monomeric level was observed and the degradates showed the molecular weights expected for the product of disulfide cleavage (see Supporting Information).

■ CONCLUSION

In conclusion, the combination of a poly(amido amine disulfide) polymer with acid-labile masking groups for targeting and stealth led to an effective siRNA delivery system with demonstrated mRNA knockdown *in vivo* in mice. Polymers used in this study were selected based on their lytic potential and monomer structure activity relationship (SAR) was identified to maximize RBC lysis activity and solubility in tandem. Selected polymers were formulated as conjugates, and those shown to have *in vitro* activity were also found to be active *in vivo* for Apo B mRNA knockdown, highlighting the utility of this screening selection strategy. Furthermore, the demonstrated ability of these polymers to degrade in the presence of glutathione illustrates their potential for biodegradability which is noted as a significant limitation of the PBAVE-based dynamic polymer conjugates reported by Rozema et al.[11] Efforts to further examine the *in vivo* efficacy and margin for these poly(amido amine) based polymers will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Additional spectra and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: rubina_parmar@merck.com, Phone: + 1-215-652-2275, Fax: +1-215-652-7310.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. J. Michael Williams of Department of Medicinal Chemistry, Merck & Co. Inc for valuable technical assistance on this research.

■ REFERENCES

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

(2) Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature 411*, 494−498.

(3) Brower, V. (2010) RNA interference advances to early-stage clinical trials. *J. Natl. Cancer Inst. 102*, 1459−1461.

(4) Davis, M. E., Zuckerman, J. E., Choi, C. H., Seligson, D., Tolcher, A., Alabi, C. A., Yen, Y., Heidel, J. D., and Ribas, A. (2010) Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature 464*, 1067−1070.

(5) Whitehead, K. A., Langer, R., and Anderson, D. G. (2009) Knocking down barriers: advances in siRNA delivery. *Nat. Rev. Drug Discovery 8*, 129−138.

(6) Sepp-Lorenzino, L., and Ruddy, M. K. (2008) Challenges and opportunities for local and systemic delivery of siRNA and antisense oligonucleotides. *Clin. Pharmacol. Therapeutics 84*, 628−632.

(7) Singha, K., Namgung, R., and Kim, W. J. (2011) Polymers in Small-Interfering RNA Delivery. *Nucleic Acid Therapeutics 21*, 133−147.

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

(9) Buyens, K., Meyer, M., Wagner, E., Demeester, J., De Smedt, S., and Sanders, N. (2010) Monitoring the disassembly of siRNA polyplexes in serum is crucial for predicting their biological efficacy. *J. Controlled Release 141*, 38−41.

(10) Murthy, N., Campbell, J., Fausto, N., Hoffman, A. S., and Stayton, P. S. (2003) Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides. *J. Controlled Release 89*, 365−374.

(11) 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., and Wolff, J. A. (2007) Dynamic polyconjugates for targeted in vivo delivery of siRNA to hepatocytes. *Proc. Natl. Acad. Sci. U. S. A. 104*, 12982−12987.

(12) Meyer, M., Dohmen, C., Philipp, A., Kiener, D., Maiwald, G., Scheu, C., Ogris, M., and Wagner, E. (2009) Synthesis and biological evaluation of a bioresponsive and endosomolytic siRNA−polymer conjugate. *Mol. Pharmaceutics 6*, 752−762.

(13) Manickam, D. S., Li, J., Putt, D. A., Zhou, Q. H., Wu, C., Lash, L. H., and Oupicky, D. (2010) Effect of innate glutathione levels on activity of redox-responsive gene delivery vectors. *J. Controlled Release 141*, 77−84.

(14) Kim, T., and Kim, S. W. (2011) Bioreducible polymers for gene delivery. *React. Funct. Polym. 71*, 344−349.

(15) van der Aa, L. J., Vader, P., Storm, G., Schiffelers, R. M., and Engbersen, J. F. J. (2011) Optimization of poly(amido amine)s as vectors for siRNA delivery. *J. Controlled Release 150*, 177−186.

(16) Kim, S. H., Ou, M., Bull, D. A., and Kim, S. W. (2010) Reductive degradation behavior of bioreducible poly(disulfide amine) for enhancing SiRNA efficiency. *Macromol. Biosci. 10*, 898−905.

(17) 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., Rohl, 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., and MacLachlan, I. (2006) RNAi-mediated gene silencing in non-human primates. *Nature 441*, 111−114.

(18) Henry, S. M., El Sayed, M. E. H., Pirie, C. M., Hoffman, A. S., and Stayton, P. S. (2006) pH-Responsive poly(styrene-alt-maleic anhydride) alkylamide copolymers for intracellular drug delivery. *Biomacromolecules 7*, 2407−2414.

(19) Chang, K. L., Higuchi, Y., Kawakami, S., Yamashita, F., and Hashida, M. (2010) Efficient gene transfection by histidine-modified chitosan through enhancement of endosomal escape. *Bioconjugate Chem. 21*, 1087−1095.