Published in final edited form as:

Acta Biomater. 2017 March 01; 50: 407–416. doi:10.1016/j.actbio.2017.01.003.

Efficient tuning of siRNA dose response by combining mixed polymer nanocarriers with simple kinetic modeling

Chad T. Greco^{a,x}, Victoria G. Muir^{a,x}, Thomas H. Epps III^{a,b}, and Millicent O. Sullivan^a

^aDepartment of Chemical and Biomolecular Engineering, University of Delaware, Newark, DE 19716, USA

^bDepartment of Materials Science and Engineering, University of Delaware, Newark, DE 19716, USA

Abstract

Two of the most prominent challenges that limit the clinical success of siRNA therapies are a lack of control over cargo release from the delivery vehicle and an incomplete understanding of the link between gene silencing dynamics and siRNA dosing. Herein, we address these challenges through the formulation of siRNA polyplexes containing light-responsive polymer mixtures, whose varied compositions and triggered release behavior provide enhanced gene silencing and controlled dose responses that can be predicted by simple kinetic models. Through the straightforward mixing of two block copolymers, the level of gene knockdown was easily optimized to achieve the maximum level of GAPDH protein silencing in NIH/3T3 cells (70%) using a single siRNA dose. The kinetic model was used to describe the dynamic changes in mRNA and protein concentrations in response to siRNA treatment. These predictions enabled the application of a second dose of siRNA to maximally suppress gene expression over multiple days, leading to a further 50% reduction in protein levels relative to those measured following a single dose. Furthermore, polyplexes remained dormant in cells until exposed to the photo-stimulus, demonstrating the complete control over siRNA activity as well as the stability of the nanocarriers. Thus, this work demonstrates that pairing advances in biomaterials design with simple kinetic modeling provides new insight into gene silencing dynamics and presents a powerful strategy to control gene expression through siRNA delivery.

Graphical abstract

^xThese authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures: The authors declare no conflicts of interest.

Supplementary Data: Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2016>.

Keywords

polyplexes; photo-responsive; siRNA dosing; block copolymers; gene silencing

1. Introduction

Therapeutic approaches exploiting RNA interference (RNAi) for post-transcriptional sequence-specific gene silencing offer unique opportunities in the treatment of a wide range of acquired and hereditary diseases.[1] Advances in the design of small interfering RNA (siRNA) have facilitated activation of this gene silencing pathway against nearly any target of interest. Additionally, the development of chemical modification strategies for the RNA backbone has conferred enhanced resistance to enzymatic hydrolysis and reduced immune responses through Toll-like receptor pathways.[2] siRNA delivery approaches already have enabled robust knockdown of aberrantly expressed genes in human clinical trials for the treatment of metastatic melanoma.[3] Furthermore, other siRNA-based approaches are under clinical investigation for the treatment of conditions as varied as diabetic macular edema and Ebola,[4] indicating the enormous promise of siRNA platforms for a range of applications in human health.

Although the use of siRNA to modulate gene expression holds tremendous promise, numerous delivery challenges have hindered clinical impact. Effective nanocarriers must satisfy seemingly contradictory demands to control binding *vs.* release of the siRNA at various points along the delivery pathway.[5] In particular, several reports have highlighted the importance of maintaining carrier stability in the presence of anionic proteoglycans and nucleases in the extracellular environment to maintain siRNA integrity.[6, 7] At the same time, additional studies have reported inefficient intracellular siRNA release as a major bottleneck facing delivery to the cytoplasm to enable interaction with the RNA-induced silencing complex (RISC).[8–11]

To overcome these challenges, many approaches have employed cationic block copolymers (BCPs) that electrostatically bind siRNA and self-assemble into nanoscale complexes (polyplexes). BCPs offer numerous appealing properties including excellent control over chain length, synthetic versatility, low dispersities, and biocompatibility.[12, 13] These unique characteristics have been harnessed to improve control over siRNA release using several different strategies. One of the most common strategies is to tune the nucleic acid binding capacity through systematically varying the molecular weight and/or charge density of the cationic block.[14, 15] Generally, increasing the number of cationic groups increases the polymer binding efficiency and cellular uptake of the polyplexes; however, increased positive charge also hinders siRNA release in the cytoplasm and results in greater

cytotoxicity of the nanocarriers.[16, 17] Most studies must compromise and use polymers with intermediate binding forces that balance the above factors to improve nucleic acid delivery.[16] Furthermore, such systematic approaches often require the synthesis of small libraries of polymers, a process that can be both tedious and costly.[18, 19]

An alternative and more flexible strategy for improving the control of siRNA release is the use of mixed polyplexes assembled from polymers with different block compositions.[20] Modulations to the net cationic charge can be achieved simply by changing the molar ratios of as few as two polymers, allowing for rapid determination of structure-function relationships.[21–23] For example, Omedes Pujol *et al.* prepared polymeric nanoparticles with varying ratios of two amphiphilic diblock copolymers that differed in their hydrophilic blocks (cationic or neutral).[24] Mixed micelles that contained greater amounts of the cationic polymer mediated higher levels of gene knockdown, but also were found to be cytotoxic, presumably due to insufficient shielding of the cationic charge in the corona.

Perhaps the most promising strategy to control siRNA release involves the use of responsive materials, whose binding affinity for siRNA can be altered by application of a stimulus.[25–27] Photo-sensitive nanocarriers offer unique advantages such as rapid response, exquisite spatial control with minimal diffusive effects, and tunability of light wavelength and intensity,[28–32] ideal for topical wound repair and other regenerative medicine applications.[33] However, although photo-responsive biomaterials have enhanced spatiotemporal control over siRNA delivery, a limited understanding of the dynamic silencing response persists.[34] RNAi depends on numerous biological parameters, such as cell doubling time, as well as mRNA and protein half-lives.[35, 36] Diseases characterized by rapid cell division, such as cancer, are difficult to treat with siRNAs because dilution effects exclude the possibility of sustained protein knockdown with a single dose.[36] Thus, multiple doses must be administered to maintain robust gene silencing over a prolonged period of time.

The majority of RNAi protocols and dosing schedules reported in the literature are chosen on the basis of precedence, or through trial and error. Multiple experiments must be conducted to screen for conditions that achieve the desired level and/or duration of knockdown, yet this tedious approach often fails to identify improved dosing regimens.[37] Kinetic modeling can provide critical insights into the underlying causes for these shortcomings. For example, Bartlett *et al.* used modeling approaches to identify a dosing frequency to induce tumor inhibition in a syngeneic mouse cancer model,[37] and these approaches later guided the design of dosing regimens employed in clinical trials.[38] Several other such models also have been published.[34–37, 39–42] However, modeling approaches typically require knowledge of numerous kinetic parameters, and modeling has most often been applied to commercial gene delivery systems (e.g. Lipofectamine, Oligofectamine, PEI),[36, 39, 42] which are incapable of precisely controlled and tunable nucleic acid activity. The development of delivery vehicles capable of externally-triggered siRNA release would provide greater versatility in the timing and magnitude of gene silencing, thereby facilitating the use of streamlined models to predict dosing schedules in regenerative medicine.

Herein, we report the use of mixtures of novel and tailorable mPEG-*b*-poly(5-(3-(amino)propoxy)-2-nitrobenzyl methacrylate) [mPEG-*b*-P(APNBMA)_n] BCPs combined with simple kinetic modeling for improved control over gene silencing. The polymers have tunable molecular weights, low dispersities, and photocleavable moieties that permit light-induced charge reversal to initiate nucleic acid release.[43] A nonfouling PEG block was incorporated to provide stability in physiological environments and resistance to opsonization. These BCPs have proven biocompatibility and protect siRNA in salt, serum, and nuclease solutions, while simultaneously stimulating siRNA release and gene-specific knockdown upon application of a cytocompatible photo-stimulus.[44]

These properties were exploited to predict and regulate siRNA dosing effects through the formulation of polyplexes containing varying ratios of two different photo-responsive mPEG-*b*-P(APNBMA)_n polymers with cationic block lengths of $n = 7.9$ and $n = 23.6$ average repeat units. By tuning polyplex composition and application of the photo-stimulus, the extent of gene silencing was easily controlled and maximized. Furthermore, temporal control over siRNA release, combined with the use of our kinetic model, facilitated the accurate prediction of dynamic changes in mRNA and protein concentrations in response to one dose or multiple doses of siRNA. Thus, we show accurate gene modulation through integration of model-based design and stimuli-responsive control over siRNA application regimens, highlighting a unique method for overcoming limitations prevalent in a variety of RNAi applications.

2. Materials and methods

2.1 Materials

The mPEG-*b*-P(APNBMA)_n ($M_n = 7,900 \text{ g mol}^{-1}$, $n = 7.9$; $M_n = 13,100 \text{ g mol}^{-1}$, $n = 23.6$) polymers were synthesized *via* atom-transfer radical polymerization as described elsewhere. [43] All siRNA molecules were purchased from GE Healthcare Dharmacon, Inc. (Chicago, IL). ON-TARGETplus non-targeting siRNAs and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNAs were used as received. Custom-made siRNA (both Dy547- and Dy647-labeled) targeting GAPDH were designed and terminally altered with 5'-P and a fluorophore (sense: 5' Dy547/Dy647-GUGUGAACACGAGAAUUAUU 3'; antisense: 5' 5'-P-UAUUUCUCGUGGUUCACACUU 3'). Dulbecco's modification of Eagle's medium (DMEM) and PBS (150 mM NaCl) were obtained from Corning Life Sciences – Mediatech Inc. (Manassas, VA). Opti-MEM® media, SuperSignal™ West Dura Chemiluminescent Substrate, and TRIzol® Reagent were purchased from Thermo Fisher Scientific (Waltham, MA). Bovine serum albumin (BSA) and a bicinchoninic acid (BCA) protein assay kit were purchased from Pierce (Rockford, IL). The anti-GAPDH and secondary HRP antibodies were purchased from AbCam (Cambridge, MA). The anti-actin antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). Primers were obtained from Eurofins MWG Operon (Huntsville, AL) with the following sequences: GAPDH forward 5' CGGGTTCCTATAAATACGGACTGC 3'; GAPDH reverse 5' CCCAATACGGCCAAATCCGT 3'; β -actin forward 5' CTGTCTGAGTCGCGTCCA 3'; β -actin reverse 5' TCATCCATGGCGAACTGGTG 3'. The iTaq™ Universal SYBR® Green

One-Step Kit and optical flat 8-cap strips were purchased from Bio-Rad (Hercules, CA). All other reagents were obtained from Thermo Fisher Scientific (Waltham, MA).

2.2 Formulation of siRNA nanocarriers

Polyplexes were formed using a self-assembly method *via* solution mixing followed by gentle vortexing. Solutions of siRNA were prepared at $32 \mu\text{g mL}^{-1}$ in 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer at pH 6.0. Polymer solutions were prepared in HEPES buffer by adding appropriate amounts of mPEG-*b*-P(APNBMA)_{7,9} and mPEG-*b*-P(APNBMA)_{23,6} to vary the fraction of each polymer in solution, on a molar basis of cationic amine groups. The polymer solutions were added to equal volumes of siRNA solutions to achieve the desired total N/P ratios (N: cationic amine groups on polymer, P: anionic phosphate groups on siRNA). Polyplexes were incubated at room temperature in a dark environment for 30 min prior to further analysis.

2.3 Ethidium bromide exclusion assay

Polyplexes were formulated as described and subjected to gel electrophoresis. Gels were prepared with 4 wt% agarose and stained with $0.5 \mu\text{g mL}^{-1}$ ethidium bromide. For analysis, 12.5 μL of polyplex solution was added to 2.5 μL of loading dye (3:7 (v/v) glycerol/water) before being added to the wells of the gel. Gels were run at 100 V for 30 min and imaged using a Bio-Rad Gel Doc XR (Hercules, CA). ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify the amounts of free siRNA by analyzing band intensities.

2.4 Cell culture and transfection

NIH/3T3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured following ATCC protocols in DMEM supplemented with 10 vol% fetal bovine serum and 1 vol% penicillin-streptomycin. The cells were maintained at 37 °C in a humidified environment with 5 vol% CO₂. For transfections, cells were seeded in 6 well plates at a density of 100,000 cells per well. The cells were allowed to adhere and recover for 24 h. In preparation for transfection, DMEM was removed, PBS was added in a wash step, and Opti-MEM® reduced serum media was added. Polyplex solutions at an N/P ratio of 4 were added to a final siRNA concentration of 20 nM, and the cells were incubated for 3 h. Following transfection, all cells were washed with PBS and incubated in fully supplemented media for 30 min. For samples undergoing 365 nm light treatment, the media was removed and replaced with Opti-MEM® without phenol red. The cells were subsequently irradiated with 365 nm light at an intensity of 200 W m^{-2} for 10 min while on a 37 °C hot plate. Subsequently, cells were incubated in fully supplemented DMEM for the remainder of the culture duration.

2.5 Protein knockdown analysis

Western blot analyses were used to measure GAPDH protein silencing. In the single dose experiments, cells were transfected as described. Then, 48 h after the start of transfection, protein was extracted from the cells by adding a lysis solution composed of 0.5 vol% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM Tris-HCl (pH 7.4), 5 mM EDTA,

and 1x Halt Protease and Phosphatase Inhibitor cocktail. For the repeated dosing experiments, a second transfection of polyplexes was performed 28 h after the first transfection. Protein was extracted *via* cell lysis 75 h after the start of the first transfection. The total protein concentration of each sample was measured using the BSA Protein Assay Kit. The protein solutions were subjected to 4% – 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 35 min at 150 V. The separated proteins then were transferred onto a poly(vinylidene fluoride) membrane at 18 V for 75 min. The membrane was subsequently blocked in 5 vol % BSA in Tris–HCl-buffered saline (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) containing 0.1 vol % Tween 20 (TBST) at room temperature for 1 h. The membrane was incubated overnight with anti-GAPDH rabbit monoclonal IgG primary antibody in TBST at 4 °C. The next day, the membrane was incubated in a solution of secondary goat anti-rabbit polyclonal IgG antibody conjugated to horseradish peroxidase (HRP) for 1 h. The SuperSignal™ West Dura Chemiluminescent Substrate was used to enable detection of the GAPDH bands through chemiluminescent imaging in a FluorChem Q (ProteinSimple, San Jose, CA), and the band intensities were quantified using ImageJ. To image the actin bands, the membrane was stripped for 15 min with Restore™ PLUS Western Blot stripping buffer, blocked in BSA solution for 1 h, and subsequently incubated with anti-actin rabbit monoclonal IgG primary antibody overnight. The next day, after incubation in a solution of secondary goat anti-rabbit polyclonal IgG antibody conjugated to HRP, chemiluminescent imaging was used to detect the actin bands.

2.6 Cellular uptake

Cells were seeded in 12-well plates at a density of 40,000 cells per well and allowed to adhere and recover for 24 h. Polyplex solutions were formed with Dy647-labeled siRNA, and these solutions were delivered to cells following the transfection protocol described previously. After the 3 h transfection, cells were washed with PBS solution and prepared for flow cytometry analysis following standard trypsin-based protocols. The cells were resuspended in PBS and filtered through a 35 µm nylon mesh to remove cell aggregates. Flow cytometry measurements were collected on an Accuri C6 instrument (BD Biosciences, San Jose, CA). The analysis was conducted at approximately 4.5 h post transfection, and at least 10,000 live cells were analyzed per sample. FlowJo v7 software (FlowJo, LLC, Ashland, OR) was used to analyze the data and quantify the mean fluorescence intensity per cell.

2.7 Light-induced siRNA release

Polyplex solutions were formed as described above, at varying fractions of mPEG-*b*-P(APNBMA)_{7,9}. Polyplexes were subsequently incubated in sodium dodecyl sulfate (SDS) solutions at an S/P ratio of 2.5 (S: sulfate groups on SDS, P: phosphate groups on siRNA) at room temperature in a dark environment for 30 min. Then, 62.5 µL of polyplex solution was sealed within a chamber composed of two glass slides with a rubber gasket. The polyplex solutions were subjected to irradiation with 365 nm light at 200 W m⁻² for 0, 5, 10, 20, or 40 min. A volume of 12.5 µL of sample was removed at each time point, and the amount of released siRNA was quantified *via* the gel electrophoresis techniques described previously.

2.8 Kinetic modeling

The dynamic silencing response was estimated with a kinetic model. A system of ordinary differential equations was used to model the changes in concentrations of siRNA, mRNA, and protein (Equations S1–3). Degradation rate constants were computed on the basis of the component half-lives reported in literature,[36, 45] and production rate constants were fit to ensure mRNA and protein steady-state values were reached in the absence of siRNA. The set of equations was solved using differential equation solver ode45 in MATLAB, and relative concentrations were normalized to 100.

2.9 Polyplex size determination

Average polyplex diameters were determined *via* fluorescence correlation spectroscopy (FCS) analysis. Polyplexes were formulated with Dy547-labeled siRNA and placed on cover slips, and the coverslips were attached to glass microscope slides made by SecureSeals from Life Technologies (Grand Island, NY). FCS measurements were performed on an LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany) using a 488 nm laser and a 40x (numerical aperture [NA] = 1.2) water immersion apochromat objective. Thirty measurements, each lasting 8 s, were taken for each sample. Data analysis was performed with ZEN 2010 software (Carl Zeiss). The structural and measurement parameters were determined using a solution of AlexaFluor555 dye with an assumed diffusion coefficient of $340 \mu\text{m}^2 \text{s}^{-1}$. [46]

2.10 Zeta potential analysis

Polyplex solutions were prepared as described and diluted with HEPES buffer to a volume of 1 mL. The solutions were transferred to a cuvette and analyzed using a ZetaPALS zeta potential analyzer from Brookhaven Instruments (Brookhaven, CT). The samples were measured at 25 °C, and the Smoluchowski model was used to analyze the data. Reported values were computed as the average of three independent experiments comprising 10 measurements each.

2.11 mRNA knockdown analysis

GAPDH mRNA knockdown was measured using qPCR. Single and double transfections were carried out as described previously, and RNA was isolated by TRIzol® Reagent, according to the manufacturer's protocols, at 48 h (single dose) and 75 h (double dose) post-transfection. The iTaq™ Universal SYBR® Green One-Step Kit and the specific GAPDH and β -actin primers were used to prepare samples for qPCR in triplicate as described in the manufacturer's protocols. The cDNA synthesis and qPCR steps were conducted on a Bio-Rad CFX96™ using the following conditions: 10 min at 50 °C; 1 min at 95 °C; 40 cycles of 10 s at 95 °C and 30 s at 60 °C; and finally, a 65 °C to 95 °C ramp at 0.5 °C every 5 s. The

CT method was used for fold change analysis,[47] and all test sample data were normalized to untreated cell data.

2.12 Statistical analysis

Results for all plots are shown as the mean $\pm$ standard deviation of data obtained from three independent samples. Statistical analyses were performed using Student's t-test or oneway

analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 siRNA binding efficiencies

Ethidium bromide exclusion assays were conducted to analyze the siRNA binding efficiencies of polyplexes formed from various ratios of mPEG-*b*-P(APNBMA)_{7.9}/mPEG-*b*-P(APNBMA)_{23.6} polymers, on a fixed molar basis of cationic amine groups. As shown in Figure 1A, formulations comprised of larger fractions of the longer polymer encapsulated siRNA more efficiently. These differences in binding affinity were apparent at N/P ratios 1 (Figure 1B). Furthermore, the ability of the nanocarriers to sequester complexed siRNA was enhanced greatly at an N/P ratio of 4, as indicated by the complete disappearance of fluorescence in the wells. Hence, all polyplexes were made at an N/P ratio of 4 in subsequent experiments to ensure complete encapsulation of siRNA and to minimize excessive positive charge.

3.2 Tuning gene silencing

Given the differences in binding affinities in the different formulations, the gene silencing efficiency of each polyplex composition was examined. Mixed polyplexes containing siRNA targeting GAPDH, a model endogenous gene, were delivered to NIH/3T3 cells, and the cells were subsequently treated with 365 nm light. As shown in Figure 2, the 0/100 polyplexes [containing only mPEG-*b*-P(APNBMA)_{23.6}] exhibited ~40% protein knockdown, consistent with the literature.[48] Interestingly, the 100/0 formulation also was able to achieve a silencing efficiency of ~40%; however, all polyplexes comprised of a mixture of the two polymers exhibited enhanced knockdown levels, demonstrating that the degree of silencing was tunable on the basis of polyplex composition. In particular, the 50/50 formulation was able to mediate gene silencing most efficiently, with ~70% protein knockdown; this silencing effect is near the highest degree achievable with a single dose of siRNA given the half-lives of the targeted mRNA and protein.[48] This simple, yet powerful, strategy of mixing BCPs produced polyplexes with greater silencing efficiencies than nanocarriers comprised of a single polymer species, implying that the polymers were functioning in a synergistic manner.

Many applications require carriers that are able not only to tune and maximize siRNA-mediated gene silencing, but also minimize off-target release and activity. As shown in Figure S1, cells transfected with any stably bound polyplex composition (e.g. an N/P ratio of 4) but not exposed to light treatment exhibited no gene knockdown. This result suggested that all of the polyplexes remained intact in the cells unless triggered by the photo-stimulus, providing on/off control of siRNA activity.

3.3 Calculating siRNA availability to predicting gene silencing

To gain an understanding of why the mixed polyplexes were able to achieve enhanced gene silencing, the siRNA availability for each formulation was estimated. Within the context of *in vitro* experiments, the major barriers to activating the RNAi process are the cellular

uptake of nanocarriers and the release of siRNA into the cytoplasm. Thus, quantitative estimates of how many siRNAs were able to overcome these two obstacles would enable the computation of siRNA availability. Kinetic modeling then could be employed to predict gene silencing efficiencies, given the relative number of siRNAs free to interact with the RISC for each formulation. Figure 3A depicts the critical biological barriers and provides an overview of our method for predicting the protein knockdown levels that were measured in Figure 2.

3.3.1 Cellular uptake of polyplexes—The relative amount of siRNAs entering the cells through cellular internalization measurements was evaluated using fluorophore-labeled nucleic acids and detection through flow cytometry. As shown in Figure 3B, the level of cellular uptake varied as a function of the polymer composition of the nanocarriers. Cellular internalization efficiencies increased as the amount of mPEG-*b*-P(APNBMA)_{7,9} in the nanocarriers increased up to 50%; however, uptake efficiencies decreased beyond this critical threshold, and the 100/0 formulation was internalized least efficiently. The 50/50 polyplexes exhibited the highest level of cellular uptake with a normalized mean fluorescence intensity (MFI) of ~160%; i.e. 60% more uptake relative to 0/100 polyplexes. The significant differences in cellular uptake were found to be dependent on both nanocarrier size and surface charge (Figure S2), and could be enhanced through the simple mixing of the BCPs.

3.3.2 Photo-triggered siRNA release—The ability of each nanocarrier to liberate siRNA upon application of the photo-stimulus was examined in SDS solutions that simulate lipid-rich intracellular environments.[48] As shown in Figure 3C, the amount of siRNA release was tunable on the basis of polyplex composition. Specifically, as the mPEG-*b*-P(APNBMA)_{7,9} content in the polyplexes increased, greater amounts of siRNA were liberated. Polyplexes containing a greater fraction of the longer polymer exhibited greater binding affinity for siRNA, which was consistent with the data in Figure 1.

3.3.3 Predicting gene silencing using kinetic modeling—Given that the levels of cellular uptake and light-induced polyplex disassembly were quantitatively determined as a function of nanocarrier composition, the relative amounts of total available siRNAs were computed for each formulation. These values were inputted into a kinetic model (Equations S1–S3) as the amount of siRNA introduced following photo-triggered nanocarrier unpackaging. The model described the dynamic changes in siRNA, mRNA, and protein concentrations on the basis of component half-lives and cellular doubling times, as described in detail elsewhere.[48] Predictions of the changes in protein expression levels were subsequently computed and are displayed in Figure 3D as red diamonds. The model accurately captured the experimentally measured gene silencing data from Figure 2 (blue bars). Specifically, the model predicted that the 50/50 formulation would exhibit the greatest level of gene silencing. All mixed polyplexes also were calculated to be more efficient than either the 0/100 or 100/0 formulations. Thus, gene silencing efficiencies can be accurately predicted *a priori* through the use of our simple kinetic model on the basis of facile siRNA release and cellular uptake measurements.

3.4 Dynamic silencing response

The kinetic model also was employed to determine when the minimum protein level for a single dose of siRNA would be reached. The 50/50 formulation was used as the basis of the model because these polyplexes exhibited the maximum knockdown efficiency (Figure 2). As shown in Figure 4A, the mRNA and protein levels maintained their steady-state values before the introduction of siRNA, which was released only after light exposure at 3.5 h post-transfection. A rapid decrease in the mRNA levels was immediately apparent, followed by the slower decrease of the protein levels. The amount of siRNA decreased with time, primarily due to dilution through cell division. Consequently, the mRNA expression levels reached a minimum level of <10% and started to recover, which caused the protein levels to plateau at ~36% (~64% silencing) before protein expression started to increase at 48 h post-transfection.

As depicted in Figure 4B, the kinetic model accurately captured the experimentally determined protein expression levels of the dynamic silencing response of the 50/50 polyplexes. Additionally, the model predicted GAPDH mRNA levels to be ~45%, which was in agreement with qPCR measurements, further validating the utility of the model. Thus, a streamlined and easily implemented kinetic modeling approach was able to accurately capture on/off silencing dynamics.

3.4.1 Dosing regimens and dynamic silencing response with multiple siRNA treatments—The model was used to predict the response to numerous dosing schedules to determine the optimal time to deliver the second dose of siRNA with the 50/50 polyplexes. Model results indicated that polyplexes delivered at 28 h post-transfection followed by 365 nm light irradiation 3.5 h later would result in significantly enhanced protein knockdown before those levels would start to recover at ~75 h post-transfection (Figure 4C). To test the validity of the mathematical model, mRNA and protein expression levels were measured at 75 h post-transfection as shown in Figure 4D. These experimentally determined values were in excellent agreement with the model results.

A noteworthy result of the above dosing studies was a reduction in GAPDH protein expression to ~15%, which is a factor of two below the minimum level achievable with a single dose (~30%). Therefore, repeated dosing was a successful method for further silencing GAPDH beyond the limits of a traditional single dose. Also, it is important to note that no gene knockdown was detected in cells that were not treated with light during the double dosing schedule, indicating that the polyplexes remained intact and the encapsulated siRNA remained dormant over several days (Figure S3). Taken together, the on/off release nature of the polyplexes, combined with the dosing schedule informed by kinetic modeling, enabled a twofold decrease in the remaining GAPDH protein levels compared to the minimum single dose level.

4. Discussion

Some of the most significant challenges that have hindered the clinical success of siRNA therapies are a lack of control over binding vs. release from the delivery vector and an insufficient understanding of the link between gene silencing dynamics and siRNA dosing.

To address these challenges, we designed mixed polymer formulations that enhanced nucleic acid release in a controllable manner, which aided in the development of accurate predictive frameworks for dosing. One of the most common strategies for tuning nucleic acid binding affinity is through the variation of the molecular weight and/or charge density of the cationic polymer.[14, 15] Generally, BCPs with longer cationic blocks package siRNA more efficiently.[16] To harness this molecular weight effect through a simpler and more scalable approach, nanocarriers were formed with various ratios of the shorter and longer mPEG-*b*-P(APNBMA) polymers. As shown in Figure 1, the amount of free siRNA at a given N/P ratio decreased as the percentage of the longer polymer in the formulation increased. Despite the three-fold difference in number of cationic groups per polymer chain, formulations consisting of only the shorter polymer were able to produce compact nanocarriers at a relatively low N/P ratio of 4. Thus, all polyplexes were formulated at an N/P ratio of 4 to minimize the number of free mPEG-*b*-P(APNBMA) polymer chains in solution.

The gene silencing capacity of each formulation was analyzed to determine whether the differences in polymer binding strengths would affect the initiation of the RNAi process. Notably, without exposure to the photo-stimulus, all polyplexes remained inactive and did not mediate any detectable gene knockdown, including the 100/0 formulation comprised entirely of the weaker binding polymer (Figure S1). The complete dormancy was surprising, given that many other siRNA delivery systems undergo rapid polyanion- or serum-induced disassembly in the extracellular environment, leading to limited cellular uptake and gene silencing efficacy.[7, 49] The result demonstrates the high stability of mPEG-*b*-P(APNBMA) polyplexes in polyanion-rich intracellular environments, likely due to cooperative electrostatic and hydrophobic interactions.[44] Robust stability is necessary to minimize undesired off-target effects in fields requiring precise spatiotemporal control over gene expression, such as regenerative medicine.[33] Furthermore, the unique stability of our polyplexes ensures that siRNA is only released on-demand, ultimately enabling precisely controlled dosing regimens.

Upon treatment with 365 nm light, the nanocarriers induced silencing that was tunable on the basis of the polyplex composition due to the synergistic effects of simply mixing BCPs (Figure 2). In particular, the 50/50 polyplexes exhibited ~70% silencing, which is approximately the highest degree of knockdown achievable with a single dose of siRNA in rapidly dividing cells due to the relatively long half-life of the GAPDH protein.[48, 50] Specifically, studies have shown that even when sufficiently large amounts of siRNA are delivered to saturate the available RISC, the activated RISC complex is diluted too rapidly by cell division to degrade all of the target mRNA.[51] Meanwhile, target proteins with a long half-life, such as GAPDH, do not fully degrade before mRNA levels begin to recover. Notably, our combined modeling and experimental results demonstrated the ability to achieve a level of silencing previously shown to correspond to RISC saturation[48] while using siRNA at a concentration of 20 nM; this concentration is significantly lower than concentrations used by many similar polyplex systems in literature (typically 100 nM).[7, 9] The efficacy of the mixed BCP polyplexes may be due to the combination of enhanced siRNA binding stability, resulting in minimal losses of siRNA to nucleases, and the ability to saturate RISC more efficiently *via* rapid siRNA release in the cytoplasm. Accordingly, we

sought to uncover the structure-function relationships underlying cellular processing and availability of siRNA.

The extent of cellular uptake is well-known to depend upon the size and zeta potential of the nanocarrier.[52–54] Furthermore, the compositions of mixed polyplex and coacervate systems play major roles in determining nanocarrier structural characteristics.[55–57] Recent reports have studied these effects with mixtures of BCPs comprised of different block constituents.[20, 24] In this work, we build on these efforts to analyze how mixing chemically-identical BCPs of different molecular weights affected nanocarrier structure.

Analysis of polyplex size revealed that nanocarriers comprised of more mPEG-*b*-P(APNBMA)_{7.9} had smaller average diameters (Figure S2). This trend is explained by the lower charge density of the shorter polymers, which have only ~33% of the number of repeat units in the cationic block but are composed of the same PEG block, as the mPEG-*b*-P(APNBMA)_{23.6} polymers. Therefore, approximately three times as many mPEG-*b*-P(APNBMA)_{7.9} chains are needed to neutralize the anionic charges of the siRNA. This self-assembly process is influenced by the inherent molecular curvature of the BCPs to minimize energetically unfavorable conditions.[58] Thus, nanocarriers with smaller diameters are favored to accommodate the extra PEG chains in the corona and reduce steric effects. It also is important to note that a single population of polyplexes was detected for each formulation, suggesting that each polyplex was comprised of proportional amounts of each polymer. Although the effects of nanocarrier size on cellular internalization depend on the nanoparticle material and cell type, smaller particles are generally thought to be beneficial for more rapid endocytic uptake.[52, 53, 59] Therefore, polyplexes with greater amounts of mPEG-*b*-P(APNBMA)_{7.9} would be expected to be internalized more efficiently.

The zeta potential of each formulation also was examined, and polyplexes comprised of higher fractions of mPEG-*b*-P(APNBMA)_{7.9} were found to have more neutral surface charges (Figure S2). This trend is the result of increased shielding of the positive polyplex core due to the inclusion of BCPs with higher mass fractions of PEG.[20, 60] For example, despite their smaller sizes, the 0/100 polyplexes exhibited lower cellular uptake efficiencies because their low zeta potentials (~2 mV) hindered electrostatically-driven transportation across the anionic cellular membrane.[54, 61] The 50/50 polyplexes balanced the opposing requirements for efficient uptake with a relatively high zeta potential of ~10 mV and a relatively small diameter of ~40 nm. Thus, we were able to optimize the nanocarrier surface charge and size *via* changes in polyplex composition to maximize cellular uptake, and ultimately overall gene silencing efficacy.

The use of mixtures of BCPs also affected the amount of siRNA release into the cytoplasm. Our analyses found that nanocarriers comprised of larger fractions of mPEG-*b*-P(APNBMA)_{7.9} disassembled to a greater extent upon application of the photo-stimulus (Figure 3C), which is consistent with reports in the literature that demonstrated polymers comprised of shorter cationic chains release nucleic acids more effectively.[14, 17] Most notably, the 100/0 polyplexes released ~10% more siRNA than the 0/100 formulations. These quantitative analyses were critical in the computation of intracellular siRNA availability, which was used in combination with kinetic modeling to accurately capture the

measured protein expression levels. It is important to note that these predictions were made possible only because our nucleic acid delivery system is capable of precise and on-demand siRNA release.

The previous analyses explained why tuning the polyplex composition synergistically altered the gene silencing efficiencies of the formulations. However, developing a deeper understanding of the dynamic nature of the RNAi process in our system would help to identify rate-limiting steps and inform methods for further improving the level of protein knockdown with the existing formulations. The dynamic silencing process is governed by fundamental biological rates, such as cell doubling time and protein and mRNA half-lives. Therefore, these parameters directly affect the maximum gene silencing efficiencies for each system with a single dose of siRNA. To demonstrate that the minimum protein levels were being measured 48 h post-transfection, kinetic modeling was employed. The characteristic rate parameters of NIH/3T3 cells and the GAPDH gene were used to generate predictions of mRNA and protein concentrations as a function of time. The model showed that 48 h post-transfection, a common time point in the literature for measuring protein knockdown, was indeed the optimal time when protein levels reached their minimum values and started to recover (Figure 4A). Furthermore, our modeling approaches showed that the silencing efficiency of our particular system was limited by two key factors. First, NIH/3T3 cells have rapid doubling times of ~24 h, which acts to dilute the intracellular siRNAs upon cell division. Second, because the GAPDH protein has a relatively long half-life, reported to be >35 h in NRK-52E rat kidney epithelial cells,[62] protein that was translated before the onset of transfection remains stable in the cell for long periods of time. Thus, within the rate-limiting constraints of our system, gene silencing was maximized through the combination of photo-responsive release and mixed polymer formulations.

The kinetic model was used to identify optimal times to measure changes in gene expression and also accurately capture the measured protein and mRNA levels (Figure 4B). Given that our approach considers only certain fundamental biological parameters, including cell doubling time and mRNA and protein half-lives, the predictions were remarkably accurate. Such simplicity is advantageous in modeling because it enables facile implementation compared to other relatively complicated models in the literature,[63] which makes it more likely to be widely adopted. Additionally, it is noteworthy that the model was able to be streamlined only because our polyplexes exhibited precise control over the timing of siRNA release.

The photo-responsive nature of our mixed polyplex system enabled the 50/50 nanocarriers to mediate the maximum level of gene silencing achievable with a single dose of siRNA, as supported through the literature and kinetic modeling. Nonetheless, some biomedical applications require genes to be knocked down below a specific threshold in order to realize therapeutic efficacy.[37] Many siRNA-based applications would benefit from prolonged silencing greater than a few days. Multiple doses of siRNA must be administered to achieve these goals, particularly in rapidly dividing cell lines. However, dosing regimens often are not implemented due to concerns with cytotoxicity or a lack of predictive capabilities to determine an optimized dosing schedule.

After generating numerous dosing regimens using the mathematical model, 28 h after the first transfection was determined to be the optimal time to deliver the second dose. This dosing schedule allowed for the introduction of a second dose of siRNA as the mRNA levels started to recover due to dilution of the first dose following cell division (Figure 4C). The model also predicted that by 75 h post-transfection, mRNA levels would start to be restored, thus resulting in increased protein expression. These predictions were remarkably accurate, as confirmed by experimental measurements of mRNA and protein levels (Figure 4D). In particular, implementation of the dosing regimen resulted in a two-fold decrease in the concentration of remaining protein. These studies demonstrate the potential of delivering multiple doses of siRNA to achieve more robust gene knockdown, which was made possible through predictions from a mathematical model.

In addition to the implementation of kinetic modeling, the straightforward mixing of BCPs made it easy to maximize silencing and reduce synthesis workloads. To demonstrate these advantages, a third mPEG-*b*-P(APNBMA)_n BCP was synthesized with an intermediate cationic block length of $n = 16.6$. As shown in Figure S4, polyplexes made with this polymer were able to mediate >62% protein silencing, which is approximately the same level of knockdown exhibited by the 50/50 mixed formulation (Figure 2). Thus, the simple mixing of BCPs with short and long cationic blocks produced formulations at least as efficient as a polymer with optimized block lengths. Moreover, this strategy bypassed the tedious and time-consuming process of polymer synthesis required to generate a library of materials.

5. Conclusions

We have demonstrated that the straightforward mixing of two photo-responsive BCPs resulted in the formulation of nanocarriers with enhanced silencing efficiencies as predicted by kinetic modeling. Upon delivery to cells, all polyplexes remained intact and did not induce protein knockdown prior to 365 nm irradiation; however, upon exposure to the photo-stimulus, the nanocarriers mediated temporally controlled gene silencing that was easily tunable on the basis of polyplex composition. Furthermore, the 50/50 formulation exhibited maximal knockdown of ~70% with a single dose of siRNA due to the saturation of cellular RNAi capacities. A simple kinetic model was employed to identify optimal double dosing schedules to further reduce GAPDH protein expression by a factor of two beyond the maximum level for a single dose. Moreover, the model confirmed that the gene silencing efficiencies were controlled by intracellular siRNA availability, as estimated through analysis of cellular uptake and light-induced siRNA release. Specifically, cellular uptake was enhanced by ~60% by optimizing nanocarrier size and zeta potential through the synergy of mixed BCPs. To our knowledge, our system is the first to mix photo-responsive, chemically-identical BCPs of different molecular weights to alter polyplex structures and improve efficacy. We anticipate that these findings and our kinetic modeling approach will be applicable to other stimuli-responsive polyplex systems with controlled siRNA release. In summary, this work uniquely combines advances in material design for improved spatiotemporal control over siRNA delivery with kinetic modeling capable of predicting gene silencing dynamics to inform optimal dosing schedules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the National Institute of General Medical Sciences of the National Institutes of Health (NIH) for financial support through an Institutional Development Award (IDeA) under grant number P20GM103541 as well as grant number P20GM10344615. The statements herein do not reflect the views of the NIH. We also acknowledge the Delaware Biotechnology Institute (DBI) and Delaware Economic Development Office (DEDO) for financial support through the Bioscience Center for Advanced Technology (Bioscience CAT) award (12A00448).

References

1. Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998; 391(6669):806–811. [PubMed: 9486653]
2. Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. *Nat. Rev. Drug Discov*. 2009; 8(2):129–138. [PubMed: 19180106]
3. Davis ME, Zuckerman JE, Choi CHJ, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD, Ribas A. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*. 2010; 464(7291):1067–1070. [PubMed: 20305636]
4. Bouchie A. Companies in footrace to deliver RNAi. *Nat. Biotechnol*. 2012; 30(12):1154–1157. [PubMed: 23222766]
5. Burke PA, Pun SH, Reineke TM. Advancing Polymeric Delivery Systems Amidst a Nucleic Acid Therapy Renaissance. *ACS Macro Lett*. 2013; 2(10):928–934. [PubMed: 24683504]
6. Zuckerman JE, Choi CHJ, Han H, Davis ME. Polycation-siRNA nanoparticles can disassemble at the kidney glomerular basement membrane. *Proc. Natl. Acad. Sci. U. S. A*. 2012; 109(8):3137–3142. [PubMed: 22315430]
7. Buyens K, Meyer M, Wagner E, Demeester J, De Smedt SC, Sanders NN. Monitoring the disassembly of siRNA polyplexes in serum is crucial for predicting their biological efficacy. *J. Controlled Release*. 2010; 141(1):38–41.
8. Shim MS, Kwon YJ. Acid-Responsive Linear Polyethylenimine for Efficient Specific and Biocompatible siRNA Delivery. *Bioconjugate Chem*. 2009; 20(3):488–499.
9. Kim HJ, Miyata K, Nomoto T, Zheng M, Kim A, Liu X, Cabral H, Christie RJ, Nishiyama N, Kataoka K. siRNA delivery from triblock copolymer micelles with spatially-ordered compartments of PEG shell siRNA-loaded intermediate layer and hydrophobic core. *Biomaterials*. 2014; 35(15):4548–4556. [PubMed: 24613051]
10. Li H-J, Wang H-X, Sun C-Y, Du J-Z, Wang J. Shell-detachable nanoparticles based on a light-responsive amphiphile for enhanced siRNA delivery. *R. Soc. Chem. Adv*. 2014; 4(4):1961–1964.
11. Gilleron J, Querbes W, Zeigerer A, Borodovsky A, Marsico G, Schubert U, Manygoats K, Seifert S, Andree C, Stoeter M, Epstein-Barash H, Zhang L, Kotliansky V, Fitzgerald K, Fava E, Bickle M, Kalaidzidis Y, Akinc A, Maier M, Zerial M. Image-based analysis of lipid nanoparticle-mediated siRNA delivery intracellular trafficking and endosomal escape. *Nat. Biotechnol*. 2013; 31(7):638–646. [PubMed: 23792630]
12. Williford J-M, Wu J, Ren Y, Archang MM, Leong KW, Mao H-Q. Recent Advances in Nanoparticle-Mediated siRNA Delivery. *Annu. Rev. Biomed. Eng*. 2014; 16:347–370. [PubMed: 24905873]
13. Kelley EG, Albert JNL, Sullivan MO, Epps TH III. Stimuli-responsive copolymer solution and surface assemblies for biomedical applications. *Chem. Soc. Rev*. 2013; 42(17):7057–7071. [PubMed: 23403471]
14. Liu X, Howard KA, Dong MD, Andersen MO, Rahbek UL, Johnsen MG, Hansen OC, Besenbacher F, Kjems J. The influence of polymeric properties on chitosan/siRNA nanoparticle formulation and gene silencing. *Biomaterials*. 2007; 28(6):1280–1288. [PubMed: 17126901]

15. deRonde BM, Torres JA, Minter LM, Tew GN. Development of Guanidinium-Rich Protein Mimics for Efficient siRNA Delivery into Human T Cells. *Biomacromolecules*. 2015; 16(10):3172–3179. [PubMed: 26324222]
16. Johnson RN, Chu DSH, Shi J, Schellinger JG, Carlson PM, Pun SH. HP MA-oligolysine copolymers for gene delivery: Optimization of peptide length and polymer molecular weight. *J. Controlled Release*. 2011; 155(2):303–311.
17. Kunath K, von Harpe A, Fischer D, Peterson H, Bickel U, Voigt K, Kissel T. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *J. Controlled Release*. 2003; 89(1):113–125.
18. Strand SP, Lelu S, Reitan NK, Davies CdL, Artursson P, Varum KM. Molecular design of chitosan gene delivery systems with an optimized balance between polyplex stability and polyplex unpacking. *Biomaterials*. 2010; 31(5):975–987. [PubMed: 19857892]
19. Nelson CE, Kintzing JR, Hanna A, Shannon JM, Gupta MK, Duvall CL. Balancing Cationic and Hydrophobic Content of PEGylated siRNA Polyplexes Enhances Endosome Escape, Stability, Blood Circulation Time and Bioactivity in Vivo. *ACS Nano*. 2013; 7(10):8870–8880. [PubMed: 24041122]
20. Miteva M, Kirkbride KC, Kilchrist KV, Werfel TA, Li H, Nelson CE, Gupta MK, Giorgio TD, Duvall CL. Tuning PEGylation of mixed micelles to overcome intracellular and systemic siRNA delivery barriers. *Biomaterials*. 2015; 38:97–107. [PubMed: 25453977]
21. Li Y, Li J, Chen B, Chen Q, Zhang G, Liu S, Ge Z. Polyplex Micelles with Thermoresponsive Heterogeneous Coronas for Prolonged Blood Retention and Promoted Gene Transfection. *Biomacromolecules*. 2014; 15(8):2914–2923. [PubMed: 24978294]
22. Wang H-X, Xiong M-H, Wang Y-C, Zhu J, Wang J. N-acetylgalactosamine functionalized mixed micellar nanoparticles for targeted delivery of siRNA to liver. *J. Controlled Release*. 2013; 166(2):106–114.
23. Wei Z, Hao J, Yuan S, Li Y, Juan W, Sha X, Fang X. Paclitaxel-loaded Pluronic P123/F127 mixed polymeric micelles: Formulation optimization and in vitro characterization. *Int. J. Pharm.* 2009; 376(1–2):176–185. [PubMed: 19409463]
24. Pujol MO, Coleman DJL, Allen CD, Heidenreich O, Fulton DA. Determination of key structure-activity relationships in siRNA delivery with a mixed micelle system. *J. Controlled Release*. 2013; 172(3):939–945.
25. Du F-S, Wang Y, Zhang R, Li Z-C. Intelligent nucleic acid delivery systems based on stimuli-responsive polymers. *Soft Matter*. 2010; 6(5):835–848.
26. Won Y-W, Yoon S-M, Lee K-M, Kim Y-H. Poly(oligo-D-arginine) With Internal Disulfide Linkages as a Cytoplasm-sensitive Carrier for siRNA Delivery. *Mol. Ther.* 2011; 19(2):372–380. [PubMed: 21081902]
27. Li J, Yu X, Wang Y, Yuan Y, Xiao H, Cheng D, Shuai X. A Reduction and pH Dual-Sensitive Polymeric Vector for Long-Circulating and Tumor-Targeted siRNA Delivery. *Adv. Mater.* 2014; 26(48):8217–8224. [PubMed: 25363160]
28. Huschka R, Barhoumi A, Liu Q, Roth JA, Ji L, Halas NJ. Gene Silencing by Gold Nanoshell-Mediated Delivery and Laser-Triggered Release of Antisense Oligonucleotide and siRNA. *ACS Nano*. 2012; 6(9):7681–7691. [PubMed: 22862291]
29. Huang X, Hu Q, Braun GB, Pallaoro A, Morales DP, Zasadzinski J, Clegg DO, Reich NO. Light-activated RNA interference in human embryonic stem cells. *Biomaterials*. 2015; 63(0):70–79. [PubMed: 26086448]
30. Shah S, Rangarajan S, Friedman SH. Light activated RNA interference. *Angew. Chem. Int. Ed.* 2005; 44(9):1328–1332.
31. Nguyen QN, Chavli RV, Marques JT, Conrad PG, Wang D, He WH, Belisle BE, Zhang AG, Pastor LM, Witney FR, Morris M, Heitz F, Divita G, Williams BRG, McMaster GK. Light controllable siRNAs regulate gene suppression and phenotypes in cells. *Biochim. Biophys. Acta-Biomembranes*. 2006; 1758(3):394–403.

32. Lee H, Kim Y, Schweickert PG, Konieczny SF, Won Y-Y. A photo-degradable gene delivery system for enhanced nuclear gene transcription. *Biomaterials*. 2014; 35(3):1040–1049. [PubMed: 24172855]
33. Saltzman WM, Olbricht WL. Building drug delivery into tissue engineering. *Nat. Rev. Drug Discov*. 2002; 1(3):177–186. [PubMed: 12120502]
34. Cuccato G, Polynikis A, Siciliano V, Graziano M, di Bernardo M, di Bernardo D. Modeling RNA interference in mammalian cells. *BMC Systems Biology*. 2011; 5:19. [PubMed: 21272352]
35. Raab RM, Stephanopoulos G. Dynamics of gene silencing by RNA interference. *Biotechnol. Bioeng*. 2004; 88(1):121–132. [PubMed: 15389485]
36. Bartlett DW, Davis ME. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res*. 2006; 34(1):322–333. [PubMed: 16410612]
37. Bartlett DW, Davis ME. Impact of tumor-specific targeting and dosing schedule on tumor growth inhibition after intravenous administration of siRNA-containing nanoparticles. *Biotechnol. Bioeng*. 2008; 99(4):975–985. [PubMed: 17929316]
38. Zuckerman JE, Davis ME. Clinical experiences with systemically administered siRNA-based therapeutics in cancer. *Nat. Rev. Drug Discovery*. 2015; 14(12):843–856. [PubMed: 26567702]
39. Varga CM, Hong K, Lauffenburger DA. Quantitative analysis of synthetic gene delivery vector design properties. *Mol. Ther*. 2001; 4(5):438–446. [PubMed: 11708880]
40. Roth CM. Quantitative measurements and rational materials design for intracellular delivery of oligonucleotides. *Biotechnol. Prog*. 2008; 24(1):23–28. [PubMed: 17887773]
41. Parra-Guillen ZP, Gonzalez-Aseguinolaza G, Berraondo P, Troconiz IF. Gene Therapy: A Pharmacokinetic/Pharmacodynamic Modelling Overview. *Pharm. Res*. 2010; 27(8):1487–1497. [PubMed: 20387096]
42. Chen HH, Ho Y-P, Jiang X, Mao H-Q, Wang T-H, Leong KW. Quantitative comparison of intracellular unpacking kinetics of polyplexes by a model constructed from quantum Dot-FRET. *Mol. Ther*. 2008; 16(2):324–332. [PubMed: 18180773]
43. Green MD, Foster AA, Greco CT, Roy R, Lehr RM, Epps TH III, Sullivan MO. Catch and release: photocleavable cationic diblock copolymers as a potential platform for nucleic acid delivery. *Polym. Chem*. 2014; 5(19):5535–5541.
44. Foster AA, Greco CT, Green MD, Epps TH III, Sullivan MO. Light-Mediated Activation of siRNA Release in Diblock Copolymer Assemblies for Controlled Gene Silencing. *Adv. Healthc. Mater*. 2015; 4(5):760–770. [PubMed: 25530259]
45. Dani C, Piechaczyk M, Audigier Y, Elsabouty S, Cathala G, Marty L, Fort P, Blanchard JM, Jeanteur P. Characterization of the transcription products of glyceraldehyde 3-phosphate-dehydrogenase gene in HeLa cells. *Eur. J. Biochem*. 1984; 145(2):299–304. [PubMed: 6499844]
46. Ross NL, Munsell EV, Sabanayagam C, Sullivan MO. Histone-targeted Polyplexes Avoid Endosomal Escape and Enter the Nucleus During Postmitotic Redistribution of ER Membranes. *Mol. Ther. Nucleic Acids*. 2015; 4:e226. [PubMed: 25668340]
47. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C-T method. *Nat. Protoc*. 2008; 3(6):1101–1108. [PubMed: 18546601]
48. Greco CT, Epps TH, Sullivan MO. Mechanistic Design of Polymer Nanocarriers to Spatiotemporally Control Gene Silencing. *ACS Biomater. Sci. Eng*. 2016; 2(9):1582–1594.
49. Vader P, van der Aa LJ, Engbersen JFJ, Storm G, Schiffelers RM. Physicochemical and Biological Evaluation of siRNA Polyplexes Based on PEGylated Poly(amido amine)s. *Pharm. Res*. 2012; 29(2):352–361. [PubMed: 21833793]
50. Malmo J, Sorgard H, Varum KM, Strand SP. siRNA delivery with chitosan nanoparticles: Molecular properties favoring efficient gene silencing. *J. Controlled Release*. 2012; 158(2):261–268.
51. Hutvagner G, Simard MJ, Mello CC, Zamore PD. Sequence-specific inhibition of small RNA function. *PLoS Biol*. 2004; 2(4):465–475.
52. Sahay G, Alakhova DY, Kabanov AV. Endocytosis of nanomedicines. *J. Controlled Release*. 2010; 145(3):182–195.

53. Hillaireau H, Couvreur P. Nanocarriers' entry into the cell: relevance to drug delivery. *Cell. Mol. Life Sci.* 2009; 66(17):2873–2896. [PubMed: 19499185]
54. Gary DJ, Puri N, Won Y-Y. Polymer-based siRNA delivery: Perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. *J. Controlled Release.* 2007; 121(1–2):64–73.
55. Grabowska AM, Kircheis R, Kumari R, Clarke P, McKenzie A, Hughes J, Mayne C, Desai A, Sasso L, Watson SA, Alexander C. Systemic in vivo delivery of siRNA to tumours using combination of polyethyleneimine and transferrin-polyethyleneimine conjugates. *Biomater. Sci.* 2015; 3(11):1439–1448. [PubMed: 26351701]
56. Wang YL, Kimura K, Dubin PL, Jaeger W. Polyelectrolyte-micelle coacervation: Effects of micelle surface charge density polymer molecular weight and polymer/surfactant ratio. *Macromolecules.* 2000; 33(9):3324–3331.
57. Bohidar H, Dubin PL, Majhi PR, Tribet C, Jaeger W. Effects of protein-polyelectrolyte affinity and polyelectrolyte molecular weight on dynamic properties of bovine serum albumin-poly(diallyldimethylammonium chloride) coacervates. *Biomacromolecules.* 2005; 6(3):1573–1585. [PubMed: 15877380]
58. Blanazs A, Armes SP, Ryan AJ. Self-Assembled Block Copolymer Aggregates: From Micelles to Vesicles and their Biological Applications. *Macromol. Rapid Commun.* 2009; 30(4–5):267–277. [PubMed: 21706604]
59. Chang T, Lord MS, Bergmann B, Macmillan A, Stenzel MH. Size effects of self-assembled block copolymer spherical micelles and vesicles on cellular uptake in human colon carcinoma cells. *J. Mater. Chem. B.* 2014; 2(19):2883–2891.
60. Lin WJ, Hsu WY. Pegylation effect of chitosan based polyplex on DNA transfection. *Carbohydr. Polym.* 2015; 120:7–14. [PubMed: 25662681]
61. Elsbahy M, Wooley KL. Design of polymeric nanoparticles for biomedical delivery applications. *Chem. Soc. Rev.* 2012; 41(7):2545–2561. [PubMed: 22334259]
62. Franch HA, Sooparb S, Du J. A mechanism regulating proteolysis of specific proteins during renal tubular cell growth. *J. Biol. Chem.* 2001; 276(22):19126–19131. [PubMed: 11262416]
63. Groenenboom MAC, Maree AFM, Hogeweg P. The RNA silencing pathway: The bits and pieces that matter. *PLoS Comput. Biol.* 2005; 1(2):155–165. [PubMed: 16110335]

Statement of Significance

Our manuscript describes two noteworthy impacts: (1) we designed mixed polymer formulations to enhance gene silencing, and (2) we simultaneously developed a simple kinetic model for determining optimal siRNA dose responses to maintain silencing over several days. These advances address critical challenges in siRNA delivery and provide new opportunities in therapeutics development. The structure-function relationships of these formulations were established to enable tuning and forecasting of nanocarrier efficiency *a priori*, leading to siRNA dosing regimens able to maximally suppress gene expression. Our advances are significant because the mixed polymer formulations provide a straightforward and scalable approach to tailor siRNA delivery regimens. Moreover, the implementation of accurate dosing frameworks addresses a major knowledge gap that has hindered clinical implementation of siRNA.

Figure 1.

Ethidium bromide exclusion analyses of mPEG-*b*-P(APNBMA) polyplexes. The polyplex composition is defined as the ratio of mPEG-*b*-P(APNBMA)_{7.9}/mPEG-*b*-P(APNBMA)_{23.6} polymers, on a molar basis of cationic amine groups. (A) Representative gel electrophoresis images of polyplexes formed at varying total N/P ratios with different polymer compositions. (B) Quantification of the gel electrophoresis images in (A) calculated from ImageJ analysis of siRNA band intensities. Results are shown as the mean $\pm$ standard deviation of data obtained from three independent experiments.

Figure 2.

GAPDH protein silencing efficiencies of mPEG-*b*-P(APNBMA) polyplexes. The polyplex composition is defined as the ratio of mPEG-*b*-P(APNBMA)_{7.9}/mPEG-*b*-P(APNBMA)_{23.6} polymers, on a molar basis of cationic amine groups. Cells were treated with 20 nM siRNA polyplexes formed at an N/P ratio of 4, irradiated with 365 nm light for 10 min, and lysed for western blot analysis 48 h post-transfection. Polyplexes are formed from various ratios of mPEG-*b*-P(APNBMA)_{7.9}/mPEG-*b*-P(APNBMA)_{23.6} polymers on a fixed molar basis of cationic amine groups. Data represent the GAPDH protein expression levels relative to the levels of the loading control β -actin, normalized to the native protein levels in controls with no siRNA treatment. Results are shown as the mean $\pm$ standard deviation of data obtained

from three independent experiments. An asterisk indicates a statistically significant difference in silencing from the 50/50 composition ($p < 0.05$).

Figure 3. siRNA release and cellular uptake analyses to predict gene silencing using kinetic modeling. The polyplex composition is defined as the ratio of mPEG-*b*-P(APNBMA)_{7.9}/mPEG-*b*-P(APNBMA)_{23.6} polymers, on a molar basis of cationic amine groups. (A) Schematic of important biological barriers that determine the level of gene silencing, as estimated through the kinetic model. (B) Cellular uptake levels as a function of polyplex composition. Fluorescently tagged siRNA was incorporated into polyplexes and delivered to cells for flow cytometry analysis. The mean fluorescence intensity (MFI) per cell was normalized to the MFI for the 0/100 polyplexes. (C) Light-triggered release of siRNA from mPEG-*b*-P(APNBMA) polyplexes. Polyplexes were formulated, incubated in SDS solutions at an S/P ratio (S: sulfates on SDS, P: phosphates on siRNA) of 2.5, and irradiated with 365 nm light

for 10 min. The solutions were analyzed by gel electrophoresis analyses, and the amount of free siRNA was quantified on the basis of relative band intensities *via* ImageJ software. (D) Kinetic modeling predictions of gene silencing on the basis of siRNA availability estimates from siRNA release and cellular uptake data. The relative amounts of delivered siRNA were computed for each polyplex composition and inputted in the mathematical model. The predicted level of protein knockdown (red diamonds) was compared to the experimentally determined values from Figure 2 (blue bars). Results for all plots are shown as the mean $\pm$ standard deviation of data obtained from three independent samples.

Figure 4.

(A & C) Kinetic models predict the dynamic nature of the GAPDH silencing process with a single dose (A) and double dose (C) of siRNA. Initial protein and mRNA concentrations were normalized to 100. A dose of siRNA was introduced 3.5 h after each transfection, which corresponds to the time of 365 nm light treatment. (B & D) Cells were lysed at 48 h or 75 h following either a single dose (B) or double dose (D) of siRNA, respectively. GAPDH mRNA and protein expression levels were determined through qPCR and western blot experiments, respectively. Model predictions of mRNA (green) and protein (orange) expression levels at the end points of 48 h and 75 h are presented as solid bars; experimental

values are presented as diagonal striped bars. Experimental values are shown as the mean $\pm$ standard deviation of data obtained from three independent samples.