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Anionic polymer and quantum dot excipients to facilitate siRNA release and self-reporting of disassembly in stimuli-responsive nanocarrier formulations

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Abstract

The incorporation of anionic excipients into polyplexes is a promising strategy for modulating siRNA binding *vs.* release and integrating diagnostic capabilities; however, specific design criteria and structure-function relationships are needed to facilitate the development of nanocarrier-based theranostics. Herein, we incorporated poly(acrylic acid) (PAA) and quantum dot (QD) excipients into photolabile siRNA polyplexes to increase gene silencing efficiencies by up to 100% and enable self-reporting of nanocarrier disassembly. Our systematic approach identified the functional relationships between gene silencing and key parameters such as excipient loading fractions and molecular weights that enabled the establishment of design rules for optimization of nanocarrier efficacy. For example, we found that PAA molecular weights ~10–20 times greater than that of the co-encapsulated siRNA exhibited the most efficient release and silencing. Furthermore, siRNA release assays and RNAi modeling allowed us to generate a PAA “heat map” that predicted gene silencing *a priori* as a function of PAA molecular weight and loading fraction. QDs further promoted selective siRNA release and provided visual as well as Förster resonance energy transfer (FRET)-based monitoring of the dynamic changes in nanostructure *in situ*. Moreover, even with the addition of anionic components, our formulations exhibited substantially improved stability and shelf-life relative to typical formulations, with complete stability after a week of storage and full activity in the presence of serum. Taken together, this study enabled synergistic improvements in siRNA release and diagnostic capabilities, along with the development of mechanistic insights that are critical for advancing the translation of nucleic acid theranostics into the clinic.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac. The information includes data from ethidium bromide exclusion assays, western blots, polyplex sizing in serum using FCS, cellular uptake studies, silencing efficiency analyses, intracellular FRET studies, and cryo-TEM micrographs. Kinetic modeling equations and a table displaying the estimated number of PAA chains per polyplex also are included.

Keywords

polyplex; photo-responsive; block copolymers; gene silencing; serum stability; poly(acrylic acid)

Introduction

RNA interference (RNAi) offers tremendous potential for the treatment of a wide range of devastating diseases.¹ Small interfering RNAs (siRNAs) can exploit this native RNAi pathway by engaging the cellular machinery to mediate post-transcriptional sequence-specific gene silencing of nearly any target of interest. Recent improvements in the design of synthetic siRNAs have provided enhanced resistance to nucleases as well as increased specificity through the reduction of off-target effects.² These advances have enabled gene knockdown in human clinical trials for the treatment of many debilitating conditions, including metastatic melanoma, transthyretin amyloidosis, and hepatitis C.^{3–5}

The high potency of siRNA is a significant potential benefit, as it means that only $\sim 10^3$ siRNAs per cell are needed to significantly suppress gene expression *in vitro* and *in vivo*.^{6–8} However, most RNAi protocols require doses corresponding to $\sim 10^7$ – 10^9 siRNAs per cell,^{9, 10} meaning that $< 0.01\%$ of the dosed siRNA molecules is available to engage the RNA-induced silencing complex (RISC), even in the most efficient delivery platforms. This inefficiency in siRNA usage is the cause of many practical issues that have hindered clinical translation, including dose-limiting toxicities and prohibitively high costs associated with administering large excesses of expensive siRNAs.^{11, 12}

One of the primary barriers to high delivery efficiencies is a lack of control over binding *vs.* release of siRNA nanopackages.^{13, 14} In particular, several reports have highlighted the importance of maintaining nanocarrier stability and siRNA binding/integrity in the presence of anionic proteoglycans and nucleases in the extracellular environment.^{15, 16} At the same time, multiple studies have demonstrated that inefficient intracellular siRNA release is a major bottleneck restricting delivery in the cytoplasm, thereby hampering siRNA interactions with RISC.^{17, 18} These contradictory requirements necessitate a compromise between stability and release in most polymeric nanocomplexes (polyplexes),¹⁹ particularly in classical two-component (siRNA/cationic polymer) systems with limited means to manipulate electrostatic interactions. Compounding these issues is a lack of diagnostic tools available to mechanistically probe binding/release behavior *in vitro* and *in vivo*.

A promising strategy for addressing these challenges is the incorporation of additional components to modulate the release of siRNA and/or enable quantification of binding/

release *in situ*.²⁰ These components often are polymers, peptides, proteins, or glycosaminoglycans with high negative charge densities^{21–23} that can alter nucleic acid binding properties, while also providing enhanced ability to release the payload from the polyplexes.^{24, 25} Specifically, anionic excipients that have a strong binding affinity for the cationic polymers may provide additional stability to the electrostatically assembled polyplexes while simultaneously enabling improved release of siRNA.^{20, 26} For example, Han *et al.* reported that the incorporation of γ -poly(glutamic acid) (γ -PGA) or sodium tripolyphosphate (TPP) into galactose-modified trimethyl chitosan-cysteine (GTC) polyplexes tuned the electrostatic interactions and increased siRNA release, resulting in enhanced gene silencing.²⁶ In addition to enhancing siRNA release, numerous studies indicate that the addition of an anionic excipient can improve biocompatibility and affect cellular uptake.²⁷ For example, the incorporation of γ -PGA into polyethylenimine (PEI)/nucleic acid complexes increased cellular internalization and reduced toxicity.²¹ Conversely, other reports demonstrated that anionic components reduce both passive uptake into cells and interactions with tissues throughout the body.^{28, 29} This stealthy behavior may be exploited *in vivo* to reduce non-specific cellular internalization and increase the delivery to the targeted tissue.^{28, 30}

The incorporation of anionic components also can provide integrated diagnostic/imaging capacities, which would be especially useful for locating and/or quantifying nanocarrier disassembly due to the innate capacity to dynamically report release events. Indeed, many promising theranostic strategies combine both therapeutic and diagnostic properties in a single formulation;³¹ however, these systems often require that the payload is labeled with the diagnostic agent³² or conjugated to a nanoparticle.³³ Both labeling and conjugation approaches can attenuate nucleic acid binding and alter intracellular trafficking in comparison to unlabeled systems.³⁴ Thus, excipients that can simultaneously enhance efficacy and provide diagnostic capabilities would avoid competitive interactions that may hinder nucleic acid activity. Such integrated approaches would accelerate testing of novel excipients to improve delivery efficiencies.

Although many excipient-containing formulations exhibited promising characteristics, several problems persist. One major challenge relates to finding a balance in binding affinity that allows for sufficient siRNA release without sacrificing extracellular stability.³⁵ For example, Huang *et al.* demonstrated that the addition of an anionic polymer, poly(glutamic acid)-*g*-poly(ethylene glycol) (PGA-*g*-mPEG), into poly(ϵ -caprolactone)-*g*-poly(N, N-dimethylaminoethyl methacrylate) (PCL-*g*-PDMAEMA)/siRNA polyplexes resulted in increased intracellular stability but reduced gene silencing efficiencies relative to unmodified polyplexes. This behavior was attributed to loosened complexes that allowed siRNA to escape prior to cellular uptake,⁹ highlighting the challenging nature of producing stable multicomponent nanocarriers. Another key hurdle in the successful use of excipients in siRNA delivery is the lack of knowledge regarding optimal molecular weights, loading fractions, and charge densities of the anionic components.³⁶ Although these properties have been studied in coacervate systems and are critical determinants of nanocarrier efficacy,³⁷ few reports have provided quantitative understanding of how these parameters affect nucleic acid delivery systems and alter siRNA release and gene silencing.^{26, 36}

In this study, we employed poly(acrylic acid) (PAA) and quantum dots (QD)s as excipients in stimuli-responsive siRNA polyplexes. PAA was chosen because of its extensive use in a variety of FDA-approved products,³⁸ high anionic charge density (almost twice that of γ -PGA),³⁶ commercial availability, and low-cost. Six different PAA molecular weights spanning three orders of magnitude were investigated to elucidate the effects of polymer chain length on polyplex stability and siRNA release. QDs coated with carboxylic acid groups were chosen due to their diagnostic capabilities (superior fluorescent properties and potential for bioimaging),³⁹ concentrated anionic charge density, and commercial availability.

PAAs and/or QDs were incorporated into polyplex assemblies comprised of mPEG-*b*-poly(5-(3-(amino)propoxy)-2-nitrobenzyl methacrylate) [mPEG-*b*-P(APNBMA)] block copolymers (BCP)s⁴⁰ and siRNA (Scheme 1). The base block copolymer consists of a nonfouling PEG block and a cationic block that undergoes charge-reversal in response to a photo-stimulus. This copolymer-based polyplex system is capable of precise photo-controlled siRNA release and gene silencing,⁴¹ making it an ideal candidate for exploring alterations in siRNA binding affinities and subtle differences in protein knockdown efficiencies.

Herein, we optimized siRNA release/gene silencing by varying the weight ratio of siRNA and PAA (with the total weight of the anionic components held constant) and the molecular weight of PAA, and we found that formulations comprised of 30/70 (w/w) PAA/siRNA with PAA molecular weights of 154 kDa and 240 kDa (an order of magnitude larger than siRNA) were the most efficient. Our PAA-containing polyplexes were extremely stable for at least one week in storage and did not lose their efficacy when transfected in serum-supplemented media. Furthermore, the gene silencing efficiency of each formulation could be predicted *a priori* on the basis of heparin-induced siRNA release assays and RNAi modeling approaches. QDs also were incorporated into the polyplexes to produce multi-functional nanostructures with increased gene silencing efficiency and diagnostic capabilities. In particular, these nanocarriers self-reported polyplex disassembly with a Förster resonance energy transfer (FRET)-based detection system, and QD incorporation enhanced visualization *via* cryogenic transmission electron microscopy (cryo-TEM). Thus, we demonstrated that incorporation of anionic excipients is a promising method for improving delivery efficiencies, reducing costs, and mitigating toxicity-associated side effects associated with nucleic acid delivery.

Experimental Section

Materials

The mPEG-*b*-P(APNBMA)_{23.6} polymer ($M_n = 13.1$ kDa) was synthesized via atom-transfer radical polymerization as described previously.⁴⁰ Non-targeted siRNA molecules (universal negative control) and branched PEI (25 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-GAPDH siRNA was obtained from GE Healthcare Dharmacon, Inc. (Chicago, IL). PAA polymers of varying molecular weights were obtained from Polysciences, Inc. (Warrington, PA) ($M_w = 2$ kDa; 30 kDa; 4,000 kDa), Polymer Source (Dorval, Quebec, Canada) ($M_w = 154$ kDa; 642 kDa), and Acros Organics (Waltham, MA) ($M_w = 240$ kDa). Qdot® 605 ITK™ Carboxyl Quantum Dots and Opti-MEM® media were

obtained from Life Technologies (Carlsbad, CA). Rabbit IgG polyclonal GAPDH antibody and secondary goat anti-rabbit IgG polyclonal horseradish peroxidase (HRP) antibody were purchased from AbCam (Cambridge, MA). The rabbit IgG polyclonal actin antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Dulbecco's Modified Eagle Medium (DMEM) and PBS (150 mM NaCl) solutions were purchased from Corning Life Sciences – Mediatech Inc. (Manassas, VA). TEM grids were obtained from Electron Microscopy Sciences (Hatfield, PA). All other reagents were purchased from Thermo Fisher Scientific (Waltham, MA).

Preparation of nanocarriers

Polyplexes were formed by a self-assembly process through mixing of siRNA, PAA, QDs and mPEG-*b*-P(APNBMA) in 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer at pH 6.0. A solution containing all anionic components (e.g., siRNA, PAA, QDs) and a solution containing the cationic mPEG-*b*-P(APNBMA) were prepared separately with equal volumes. The standard (unmodified) polyplex was comprised of 0.4 μ g siRNA and mPEG-*b*-P(APNBMA) at an N/P (N: amine groups on mPEG-*b*-P(APNBMA); P: phosphate groups on siRNA) of 4 in a total volume of 25 μ l. In PAA-modified polyplexes, the PAA/siRNA weight ratios were varied while holding the total weight of the anionic components constant. (*Note: the compositions of PAA-containing polyplexes are reported as PAA/siRNA (w/w) ratios.*) The cationic block copolymer solutions were added dropwise to the anionic component solutions while mixing thoroughly *via* vortexing. The resulting solutions were incubated at room temperature for 30 min prior to further analysis.

Cell culture

Mouse embryonic fibroblasts (NIH/3T3) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and were cultured following ATCC protocols in a growth medium consisting of DMEM supplemented with 10 vol% fetal bovine serum (FBS) and 1 vol% penicillin-streptomycin (P/S). The cells were cultured in a humid environment maintained at 37 °C and 5 vol% CO₂.

In vitro cell transfection

NIH/3T3 cells were cultured in six-well plates at a density of 10,000 cells cm⁻² for 24 h. Before transfection, the supplemented growth medium was removed, and the cells were washed with PBS. Opti-MEM was added to the plates before the polyplex solutions were added dropwise to a final concentration of 20 nM in each well. Following a 3 h transfection period, the Opti-MEM was replaced with supplemented DMEM for a 30 min recovery period. The DMEM was replaced with phenol red-free Opti-MEM®, and the cells were irradiated with 365 nm light at an intensity of 200 W/m² for 10 min on a 37 °C hotplate. Supplemented DMEM was added to the wells after irradiation.

GAPDH protein knockdown

Cells were cultured for 48 h following transfection. The cells were lysed with a solution consisting 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 cocktails.

The total protein concentration for each sample was determined with a BCA Protein Assay Reagent Kit, and the samples were subjected to 4% – 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis for 35 min at 150 V. The protein was transferred to a poly(vinylidene fluoride) membrane at 18 V for 70 min, and the membrane was subsequently blocked in 5 vol% bovine serum albumin (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 in a solution of anti-GAPDH rabbit monoclonal IgG primary antibody ($0.5 \mu\text{g mL}^{-1}$ in TBST) at 4°C overnight. Afterwards, the membrane was incubated with a solution of goat anti-rabbit polyclonal IgG antibody conjugated to HRP ($0.4 \mu\text{g mL}^{-1}$ in TBST) at room temperature for 1 h. Target proteins were visualized using SuperSignal™ West Dura Chemiluminescent Substrate with a FluorChem Q imager (ProteinSimple, San Jose, CA). Next, antibodies were stripped from the blot using Restore™ PLUS Western Blot stripping buffer for 15 min. The membrane was blocked in a 5% BSA solution for 1 h and then incubated in anti-actin rabbit polyclonal IgG ($0.3 \mu\text{g mL}^{-1}$ in TBST) overnight. The next day, after incubation with a solution of secondary goat anti-rabbit polyclonal IgG antibody conjugated to HRP, chemiluminescent imaging was used to detect the actin bands. The band intensity of each target protein was analyzed with ImageJ software.

Serum and storage stability assay

Polyplexes were formed either 30 min or 1 week prior to transfection. The GAPDH protein knockdown protocol described above was followed, except that transfections were carried out in DMEM containing 10 vol% FBS and 1 vol% P/S for serum stability experiments.

Heparin-induced siRNA release

Heparin was added to polyplex samples at a weight ratio of 5:1 (heparin:combined weight of siRNA and PAA). The solutions were gently vortexed and allowed to incubate in the dark at room temperature for 30 min. A 6x loading dye [3/7 (v/v) glycerol/water] was added to the polyplex solutions, and the solutions were subsequently loaded into a 2 wt% agarose gel containing $0.5 \mu\text{g mL}^{-1}$ ethidium bromide and run at 100 V for 30 min. The wells were imaged on a Bio-Rad Gel Doc XR, and the intensity of each free siRNA band was quantified using ImageJ software and compared to a control band of free siRNA.

RNAi modeling

A set of equations (Equations S1–3) was solved using differential equation solver ode45 in MATLAB. The initial concentrations of siRNA were varied according to the heparin-induced siRNA release data.

FRET-based polyplex disassembly

Polyplexes were formulated as described with either 2 nM QD605 and 10 nM Dy647-labeled siRNA (for QD-containing polyplexes) or 10 nM Dy547- and 10 nM Dy647-labeled siRNA (for polyplexes not containing QDs). Following the light treatment procedure detailed above, polyplex solutions were loaded into cuvettes. The fluorescence emission

(525–750 nm) was measured on a Fluoromax-4 fluorescence spectrophotometer (Horiba, Kyoto, Japan) using an excitation wavelength of 488 nm.

Cryogenic transmission electron microscopy (cryo-TEM) imaging of nanocarriers

Polyplexes were formulated with 30/70 PAA/siRNA (240 kDa) and 1 nM QDs as described above. The formulations were air-dried until the solutions were ~10x concentrated. Cryo-TEM samples were prepared using a FEI Vitrobot. Before loading, carbon-coated copper TEM grids (C-flat™ holey carbon film with 200 mesh and Multihole patterning) were cleaned by plasma etching for 60 s. A 5 µL drop of polyplex solution was added to the grid, and the grid was blotted twice to remove the excess solution. The grids were plunged into cold liquid ethane to vitrify the sample and then transferred to liquid nitrogen. The grids were transferred to a Gatan cryotransfer holder and imaged with a FEI Talos TEM operating at 120 kV. The images were analyzed using DigitalMicrograph Version 3.9.4 and ImageJ Version 1.47.

Results and Discussion

Formulation of PAA-containing polyplexes

Ternary complexes were formulated with varying weight fractions of siRNA and PAA to determine how much of the cationic polymer was needed for complete encapsulation of siRNA in the presence of both siRNA and the PAA excipient. Ethidium bromide exclusion assays showed that formulations made with larger fractions of PAA were not able to encapsulate siRNA as efficiently (Figure S1). Specifically, whereas siRNA in the 0/100 (PAA/siRNA) formulation was completely bound in polyplexes at an N/P ratio of 1, consistent with earlier studies,⁴¹ free siRNA remained in the 30/70 and 50/50 formulations at an N/P ratio of 2 and was not completely encapsulated in these samples until an N/P of 4. Thus, all nanocarriers were formulated at an N/P ratio of 4 in subsequent studies to ensure complete encapsulation. The decrease in siRNA binding affinity upon PAA addition likely was a result of the higher anionic charge density of PAA,³⁶ resulting in lower effective charge ratios. In particular, the effective charge ratios of the 0/100, 10/90, 30/70, and 50/50 formulations are ~4.0, ~3.0, ~2.0, and ~1.4, respectively. The high anionic charge of PAA is favorable for inducing greater amounts of siRNA release intracellularly because it leads to preferential retention of PAA relative to the siRNA.²⁶

Gene silencing of PAA-containing polyplexes

Given that PAA incorporation altered siRNA binding affinity, we investigated how the polyplex composition affects gene silencing. To this end, the molecular weight of the PAA was varied over three orders of magnitude at various PAA loading fractions. Following transfection, cells were treated with a photo-stimulus, and the protein silencing efficiency was measured. The fold change in silencing efficiency per weight of siRNA delivered was compared to control polyplexes formulated without PAA. As shown in Figure 1, all 10/90 formulations exhibited no significant differences in gene silencing in comparison to the control polyplexes. Polyplexes comprised of 50/50 PAA/siRNA also had protein knockdown levels comparable to 0/100 PAA polyplexes, although the efficacy of these formulations was more dependent on PAA molecular weight. Conversely, the 30/70 polyplexes comprised of

154 kDa and 240 kDa PAA demonstrated enhanced gene silencing that was nearly 100% more efficient than the polyplexes without PAA (2-fold difference). Within each loading fraction, the 4,000 kDa PAA formulations generated the least efficient silencing responses, and the intermediate PAA molecular weights provided the highest levels of knockdown. It is important to note that all formulations remained dormant and did not mediate protein silencing in the absence of 365 nm light (Figure S2). The on/off photo-controlled nature of the polyplexes is indicative of their intracellular stability and provides a distinct advantage over other polyplex systems for biomedical applications that require precise spatiotemporal control over gene expression.⁴²

Serum and storage stability

The most efficient formulation (30/70, 240 kDa) was analyzed further to elucidate the serum and storage stability of the polyplexes. First, polyplexes were exposed to either serum-free media or serum-supplemented media to determine if the polyplexes could resist serum-induced aggregation or degradation and maintain activity. FCS analyses showed that there was no significant change in the average polyplex diameter following incubation in FBS for 3 h (Figure S3). As shown in Figure 2A, the mPEG-*b*-P(APNBMA)/PAA polyplexes mediated the same level of knockdown in serum-supplemented and serum-free media. However, polyplexes made with PEI, a cationic polymer commonly used in nucleic acid delivery, rapidly aggregated and were ineffective in silencing gene expression (Figure 2B). Furthermore, mPEG-*b*-P(APNBMA)/PAA polyplexes that were formulated and stored for one week prior to transfection exhibited similar gene silencing efficiencies as polyplexes used 30 min prior to transfections in serum-free media. mPEG-*b*-P(APNBMA)/PAA polyplexes that were stored for one week prior to transfection in serum-supplemented media mediated a higher gene silencing efficiency than PEI and mPEG-*b*-P(APNBMA) polyplexes without PAA in standard conditions (30 min storage, serum-free transfection media).

Polyplex stability in polyanion-rich environments, such as serum-containing culture media, is a particularly important problem that has limited translation of nucleic acid delivery vehicles.³⁵ Although many nanocarriers are able to resist aggregation and maintain their size in serum, protein-nanoparticle interactions remain a major challenge.⁴³ For example, Sizovs *et al.* formulated a number of nanocomplexes that exhibited promising serum stability properties, but most failed to mediate gene knockdown in serum-supplemented media.⁴⁴ This phenomenon is representative of the widespread problem of biotransformation, a process by which proteins and other biomolecules adsorb to nanocarrier surfaces, forming a corona that critically alters the physico-chemical identity of the nanostructure *in vivo*.⁴⁵ These changes may inhibit cellular internalization, induce premature polyplex disassembly, or trigger activation of the immune system.^{44, 46} The fact that mPEG-*b*-P(APNBMA)/PAA polyplexes did not lose efficacy in serum-supplemented media suggests that these formulations are extremely stable and may be well suited for *in vivo* applications.

The ability of nanocarriers to remain stable over relatively long periods of time is another critical characteristic required for clinical use.⁴⁷ This feature is particularly important in settings that require numerous doses to be administered over multiple days, as a single batch of nanocarriers can be formulated and used for all doses. However, in the literature, the vast

majority of polyplexes are formulated within a few hours of their desired use to minimize aggregation over time. Moreover, most polyplex systems capable of prolonged stability often require lyophilization or storage in non-ambient conditions, which hinders practical application.⁴⁸

The fact that our nanocarriers were able to mediate efficient gene silencing in serum-supplemented media following storage for one week is indicative of their robust stability in biological environments. In addition to the incorporation of an anti-fouling PEG block in mPEG-*b*-P(APNBMA), hydrophobic interactions between the aromatic rings in the cationic block and the siRNA backbone likely confer enhanced resistance to biotransformation.⁴¹ Multiple reports also have demonstrated that formulating polyplexes with a combination of hydrophobic and electrostatic interactions improved *in vivo* stability and gene silencing.^{18, 49} Nevertheless, subsequent studies were conducted in Opti-MEM to isolate the potential effects of heparin-induced siRNA release on gene silencing efficiencies.

Heparin-induced siRNA release and cellular uptake

The ability of anionic glycosaminoglycans, such as heparin, to mediate nucleic acid release from nanocarriers has been shown to be correlated with transfection efficiencies.⁵⁰ To this end, heparin stability assays were conducted to better understand why the various mPEG-*b*-P(APNBMA)/PAA polyplexes mediated different levels of gene silencing. Following addition of heparin at a 5:1 heparin:siRNA weight ratio, the percent of siRNA that was liberated from each formulation was measured by gel electrophoresis as shown in Figure 3. Generally, 10/90 and 50/50 polyplexes released smaller percentages of siRNA than 30/70 polyplexes. Formulations made with the highest molecular weight PAA (4,000 kDa) released the smallest fractions of siRNA, and formulations made with intermediate molecular weights (154 kDa and 240 kDa) disassembled to the greatest extent, particularly in the case of 30/70 polyplexes. Therefore, a range of PAA molecular weights and weight fractions was identified that exhibited the greatest amounts of siRNA release, consistent with gene silencing studies. Notably, although PAA was incorporated to increase gene silencing by reducing polymer-siRNA binding, mPEG-*b*-P(APNBMA)/PAA polyplexes remained sufficiently stable as to require high heparin:siRNA ratios to induce detectable siRNA release, in distinct contrast to PEI-siRNA polyplexes.⁴¹

The gene silencing trends reported in Figure 1 are remarkably similar to the siRNA release data shown in Figure 3, suggesting that the modulation of binding *vs.* release was a key factor dictating knockdown efficacy. In particular, formulations comprised of intermediate PAA weight fractions (30/70 PAA/siRNA) and intermediate PAA molecular weights (154–204 kDa) released the greatest amounts of siRNA and mediated the most efficient gene knockdown. These trends are influenced by a number of factors including excipient chain length, overall nanocarrier charge, and number of excipient molecules per nanocarrier. All 10/90 formulations exhibited silencing efficiencies similar to the control polyplexes without PAA, suggesting that a 10 wt% modification is not enough to significantly alter the physical properties of the nanocarriers. However, formulations that had 50 wt% of the siRNA replaced with PAA also did not mediate significantly improved gene silencing, which is a result of PAA having an anionic charge density approximately five times greater than

siRNA. In particular, these nanocarriers had overall charge ratios that were close to neutral (effective charge ratio of ~ 1.4), which decreases the competitive binding interactions with intracellular polyanions that promote polyplex disassembly. The 30/70 formulations, on the other hand, possessed almost twice as many cationic charged as anionic charges (effective charge ratio of ~ 2.0) and strongly interacted with polyanions, such as heparin, to liberate siRNA.

For all weight fractions of PAA, but particularly in the case of 30/70 polyplexes, the optimal PAA molecular weights were approximately 10–20 times greater than the molecular weight of siRNA (~ 13 kDa). Many reports in the literature have demonstrated that the binding affinity of ionic polymers increases with molecular weight.^{19, 51} As the PAA molecular weight increases to molecular weights much greater than the molecular weight of siRNA, the ionic interactions are stronger and the PAA preferentially binds to the cationic polymer so that siRNA can be selectively released. At the same time, the longer PAA chains can form entanglements within the nanocarriers that stabilize the polyplexes and limit their disassembly.^{24, 52} Additionally, if the weight fraction of PAA is held constant, the number of individual polymers in each polyplex decreases as the molecular weight of PAA increases (Table S1). For example, the 30/70 4,000 kDa PAA nanocarriers would have ~ 0.3 PAA chains per polyplex, meaning that the majority of the nanocarriers are unmodified and contain no PAA. This analysis helps explain why this particular formulation exhibited gene silencing efficiencies similar to the control polyplexes without PAA. Taken together, PAA polymers with intermediate molecular weights balance all of these effects (i.e., providing preferential binding, limiting the amount of chain entanglement, and distributing PAA chains in all polyplexes) more effectively than the high and low molecular weight polymers.

In addition to intracellular siRNA release, cellular uptake also may play a significant role in determining overall efficiency.²⁵ Thus, cellular uptake analyses were conducted to investigate the effect of polyplex composition on nanocarrier internalization. The amount of siRNA uptake was proportional to the weight fraction of siRNA in the formulations (Figure S4), indicating that approximately the same number of polyplexes were being internalized regardless of the amount of PAA incorporation. Therefore, differences in cellular uptake likely do not significantly contribute to the differences in gene silencing efficiency.

Predicting gene silencing *via* siRNA release assays

To verify that the gene silencing efficiency of each formulation was primarily controlled by the amount of siRNA the delivery vehicles could liberate, the siRNA release data from Figure 3 was inputted into a kinetic model of siRNA-mediated gene silencing established previously (Equations S1–3).⁵³ As shown in Figure 4, the model predicted gene silencing levels that were similar ($< 15\%$ for nearly all polyplex compositions) to those measured experimentally in Figure 1. The ability of this method to capture the complex process of gene silencing was particularly exciting given the simplicity of the heparin stability assays and kinetic modeling. The accuracy of the predictions also suggested that siRNA release and protein/mRNA turnover, as opposed to other considerations (e.g. the effects that the polymer chains may have on RISC interactions with siRNA), were the rate-determining steps in our system. Furthermore, use of the kinetic model enabled generation of a “heat map” to identify

the amount and molecular weight of excipient *a priori* for achieving a desired level of knockdown. These insights into PAA compositions are likely directly translatable to other anionic polymer excipients and stimuli-responsive polyplex systems. For example, a recent study screened numerous anionic excipients and found that the most effective formulation at inhibiting tumor growth in an *in vivo* mouse model contained an anionic polymer (γ -PGA) with a molecular weight within the ideal range determined herein.²⁶ The authors partially attributed this enhancement to chain entanglement effects but did not vary the γ -PGA loading fraction to explore the effect of number of charged groups on polyplex efficacy. Thus, our streamlined approach to optimizing polyplex compositions may improve current systems and facilitate the introduction of other excipients more rapidly and efficiently.

Incorporation of quantum dots: selective siRNA release

In addition to PAA, other anionic components can be added to enhance the selective release of siRNA. Inclusion of QDs, which are ideally suited for fluorescence-based analyses and are amenable to bioimaging techniques,³⁹ may allow for the development of theranostic nanocarriers. Various concentrations of QDs were added to mPEG-*b*-P(APNBMA)/PAA polyplexes during formulation, as depicted in Figure 5A. Polyplexes incorporating PAA were comprised of 30/70 PAA/siRNA (240 kDa PAA), the most efficient formulation identified in Figure 1. Heparin-induced siRNA release assays were conducted and visualized using gel electrophoresis. As shown in Figure 5B, increasing amounts of siRNA were released as the concentration of QDs increased. Moreover, selective release of siRNA was exhibited for QD concentrations of 0.5–2 nM and 0.5–1 nM for the 0/100 and 30/70 polyplexes, respectively, as indicated by the lack of free QDs migrating down the gel. The percent of siRNA released was quantified and is displayed in Figure 3C. The siRNA release efficiency was higher across all QD concentrations for the 30/70 polyplexes relative to the polyplexes without PAA. The siRNA release data was inputted into our RNAi model (Equations S1–3) to predict protein knockdown levels, which were found to be in agreement with experimental data (Figure S5). Thus, inclusion of additional anionic components increased the selective release of siRNA and enhanced the gene silencing efficiency.

QD-based FRET analyses of polyplex unpackaging

Monodisperse QDs have unique fluorescence properties, such as high photo-stability and narrow symmetric emission spectra, that make them ideal candidates for use in FRET-based analyses.³⁹ Polyplexes were formulated with various combinations of QDs, fluorescently labeled siRNA, and PAA before being irradiated with light for various lengths of time. The FRET ratio, defined as the ratio between the peak intensities of the donor (QD or Dy547-siRNA) and acceptor (Dy647-siRNA) fluorophores, was computed and is plotted in Figure 6A. Control polyplexes with no modifications lost the FRET signal the slowest, indicating that greater amounts of siRNA and QDs remained tightly packaged in the nanocarriers following irradiation. All other formulations lost at least 90% of the FRET signal after 10 min of irradiation, indicating that the polyplexes loosened and that the average distance between siRNAs and QDs was at least 44% greater than the Förster radius (~9 nm).⁵⁴ Polyplexes modified with PAA disassembled more rapidly than polyplexes modified with QDs; however, polyplexes incorporating both QDs and PAA disassembled the fastest. These

trends are particularly apparent following 10 min of irradiation, which is the duration of light treatment applied during transfections, as plotted in Figure 6B.

The fluorescence-based diagnostic capabilities of QDs also can easily be extended to live cells to enable analysis of polyplex disassembly. As shown in Figure S6, mPEG-*b*-P(APNBMA)/PAA (30 wt% 240 kDa)/QD (2 nM)/Dy647-siRNA polyplexes were formulated and transfected into NIH/3T3 cells. Following irradiation with 365 nm light for varying lengths of time, the FRET fluorescence intensity was measured using flow cytometry. Cells that were not irradiated with light exhibited strong fluorescence signals, and the fluorescence intensity decreased as the cells were irradiated with the photo-stimulus for longer times (Figure S6A). After 10 min of irradiation, ~95% of the FRET signal was lost (Figure S6B). The dynamics of polyplex loosening/disassembly in solution (Figure 6) were relatively consistent with those detected intracellularly (Figure S6), further indicating that siRNA release measurements in solution are a reasonable predictor of polyplex behavior inside cells.

Recently, the use of fluorescence-based imaging agents has become an essential tool in the fields of drug delivery and bioimaging.⁵⁵ The incorporation of QDs into the mPEG-*b*-P(APNBMA) complexes enabled the formation of nanocarriers that can self-report on their dynamic structural changes and disassembly in solution. Such information is critical for accessing the effectiveness of various excipients in nanocarriers, which can be used to predict gene silencing efficiencies using modeling methods discussed above. Given the excellent serum stability of the mPEG-*b*-P(APNBMA) complexes, the QD-containing nanocarriers may serve as effective *in vivo* fluorescence-based imaging probes.

Cryo-TEM imaging of QD-containing polyplexes

In addition to their advantageous fluorescence properties, QDs also hold tremendous promise as diagnostic imaging agents for tracking delivery vehicles in live cells and *in vivo*.⁵⁶ The high electron density of QD cores provides better contrast relative to polymeric/nucleic acid systems, which often are barely visible due to their hydrated nature and low electron density.⁵⁷ Many types of electron microscopy are useful for studying nanoparticle distribution in tissue samples;^{6, 58} however, cryo-TEM is uniquely suited for the analysis of nanocarriers in solution prior to delivery into biological samples. This powerful technique allows for the imaging of solution-assembled nanostructures in their native environment without the need for the samples to be stained or fixed.⁵⁸ Specifically, cryo-TEM avoids many challenges associated with dry state TEM and atomic-force microscopy such as solvent evaporation, surface interactions, chemical fixation, and staining, all of which may lead to structural changes and/or artifacts.⁵⁹

To access the bioimaging potential of the formulations, mPEG-*b*-P(APNBMA)/PAA/QD siRNA complexes were prepared and imaged using cryo-TEM (Figure 7). The QDs were clearly visible as dark spheres with diameters of 5–10 nm. Moreover, the QDs were clustered within polymer/siRNA complexes that appeared as a light shade relative to the QDs due to the low electron densities of mPEG-*b*-P(APNBMA) and siRNA (Figure S7A). The polyplexes had a consistent spherical-like morphology with average diameters of ~150 nm (Figure S7B), which is in agreement with the average sizes obtained using FCS (Figure

S3). FCS analyses previously showed that ~250 siRNAs were incorporated into each polyplex.⁵³ Given that the siRNA was used at a 20x molar excess to the 1 nM QDs, we estimated that ~13 QDs would be included within each complex; the representative polyplexes imaged in Figure 7 and Figure S7 corroborated this estimate. Gel electrophoresis experiments verified that no QDs were free in solution (see also Figure 5 and associated text with Figure S7). Without the high contrast provided by the QDs, the polymeric polyplexes would have been extremely difficult to distinguish (Figure S7C). Thus, the incorporation of QDs facilitated the bioimaging of polyplexes, which is an indispensable tool for tracking nanocarriers in diagnostic applications.

Conclusions

In summary, we systematically explored the incorporation of PAA and QD excipients in photo-responsive complexes to enhance the utilization of siRNA. Wide ranges of PAA loading fractions and molecular weights were investigated, and the most efficient compositions were 30/70 PAA/siRNA with PAA molecular weights of 154 kDa and 240 kDa (~10–20 times that of siRNA). These nanocarriers mediated selective siRNA release and improved the gene silencing efficiency by 100% relative to unmodified polyplexes. Moreover, the nanocarriers maintained full activity either when stored for one week or when transfected in serum-supplemented media. siRNA release studies enabled the generation of a predictive “heat map” that provided *a priori* insight into gene silencing efficiencies as a function of PAA loading fraction and molecular weight. These parameters governed polyplex activity on the basis of charge density and entanglement effects. QDs also were incorporated into polyplexes, and formulations containing 2 nM QDs enhanced gene silencing efficiency by 40% relative to unmodified polyplexes. Furthermore, the QD-containing polyplexes were able to self-report dynamic structural changes and nanocarrier disassembly through fluorescence-based analyses, and cryo-TEM analyses demonstrated that QDs enhanced the bioimaging potential of the nanocarriers. Thus, this work highlights the ability of excipients to improve the efficiency of nucleic acid delivery vehicles and develop theranostics capable of self-reporting their disassembly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Silencing efficiency fold change of mPEG-*b*-P(APNBMA)/PAA ternary siRNA polyplexes. siRNA was substituted with PAA on a weight percent basis, and the molecular weight of PAA was varied. Cells were treated with 20 nM siRNA polyplexes, irradiated with 365 nm light for 10 min, and lysed for western blot analysis at 48 h post-transfection. Data represent the fold change in GAPDH protein silencing efficiency (on a per µg of siRNA basis) of each formulation relative to polyplexes without PAA (fold change = 1). Results are shown as the mean of data obtained from three independent experiments.

Figure 2.

Polyplex stability in serum-containing media and following storage for one week. The silencing efficiencies of (A) mPEG-*b*-P(APNBMA)/PAA (30/70, 240 kDa) ternary siRNA polyplexes and (B) PEI/siRNA polyplexes were analyzed. Transfections were conducted in either OptiMEM (serum-free) or DMEM containing 10% FBS. Polyplexes were incubated for either 30 min or 1 week prior to transfection. Cells were treated with 20 nM siRNA polyplexes, irradiated with 365 nm light for 10 min, and lysed for western blot analysis at 48 h post-transfection. Data represent the fold change in GAPDH protein silencing efficiency (on a per μg of siRNA basis) of each sample relative to polyplexes without PAA (red dashed line). Results are shown as the mean $\pm$ standard deviation of data obtained from three independent experiments. An asterisk indicates a statistically significant difference between a given sample and polyplexes incubated for 30 min prior to transfection in serum-free media ($p < 0.05$).

Figure 3.

Heparin-induced siRNA release from mPEG-*b*-P(APNBMA)/PAA ternary siRNA polyplexes. siRNA was substituted with PAA on a weight percent basis, and the molecular weight of PAA also was varied. Following polyplex formulation, heparin was added at a weight ratio of 5:1 heparin:siRNA and incubated for 30 min. The solutions were subjected to gel electrophoresis and the amount of free siRNA was quantified using ImageJ. Results are shown as the mean of data obtained from three independent experiments.

Figure 4.

Model predictions of silencing efficiency fold change of mPEG-*b*-P(APNBMA)/PAA ternary siRNA polyplexes. The relative amounts of heparin-induced siRNA release (Figure 2) were inputted into the RNAi model to predict the fold change in GAPDH protein silencing efficiency (on a per μg of siRNA basis) of each formulation relative to polyplexes without PAA (fold change = 1).

Figure 5.

Heparin-induced siRNA release from polyplexes incorporating QDs. Varying amounts of QDs were added during mPEG-*b*-P(APNBMA)/PAA/QD siRNA polyplex formation. Following complex formulation, heparin was added at a heparin:siRNA weigh ratio of 5:1 and incubated for 30 min. (A) Cartoon schematic of polyplex formation. (B) The solutions were subjected to gel electrophoresis. (C) The amount of free siRNA from the agarose gels was quantified using ImageJ. Results are shown as the mean $\pm$ standard deviation of data obtained from three independent experiments.

Figure 6.

Light-induced polyplex disassembly of mPEG-*b*-P(APNBMA) polyplexes. The complexes were formulated with various combinations of 2 nM QDs, 30 wt% PAA, and fluorophore-labeled siRNA. Following irradiation with 365 nm light for varying lengths of time, the fluorescence intensity was measured. (A) The FRET efficiency was computed for each complex composition as follows: no modifications (green), QDs (blue), PAA (purple), and QDs and PAA (red). Results are shown as the mean $\pm$ standard deviation of data obtained from three independent experiments for each time point. (B) The FRET ratio following 10 min of irradiation. The results are reported as the average fluorescence of three independent samples for each irradiation time. *Note: all of the groups (at 10 min) are statistically different from each other ($p < 0.05$).*

Figure 7.

Representative visualization of an isolated QD-containing polyplex using cryo-TEM. A solution of mPEG-*b*-P(APNBMA)/PAA (30 wt% 240 kDa)/QD (1 nM) siRNA polyplexes was formulated and prepared for cryo-TEM analyses. The white circular line indicates the approximate edge of the polyplex to guide the eye. The dark 5–10 nm spheres are QDs, which appear confined within the polyplex. The scale bar represents 100 nm.

Scheme 1.

Cartoon schematic of mPEG-*b*-P(APNBMA) siRNA polyplexes (A) without PAA or QDs and (B) with PAA and QDs. Following polyplex formation, the formulations were treated with a photo-stimulus to trigger unpacking of siRNA. The incorporation of the excipients resulted in enhanced and selective release of siRNA.