Published in final edited form as:

ACS Nano. 2017 June 27; 11(6): 5680–5696. doi:10.1021/acsnano.7b01110.

Zwitterionic Nanocarrier Surface Chemistry Improves siRNA Tumor Delivery and Silencing Activity Relative to Polyethylene Glycol

Meredith A Jackson, Thomas A. Werfel, Elizabeth J. Curvino, Fang Yu, Taylor E. Kavanaugh, Samantha M. Sarett, Mary D. Dockery, Kameron V. Kilchrist, Ayisha N. Jackson, Todd D. Giorgio, and Craig L. Duvall

Department of Biomedical Engineering, Vanderbilt University, Nashville, Tennessee 37240, United States

Abstract

Although siRNA-based nanomedicines hold promise for cancer treatment, conventional siRNA–polymer complex (polyplex) nanocarrier systems have poor pharmacokinetics following intravenous delivery, hindering tumor accumulation. Here, we determined the impact of surface chemistry on the *in vivo* pharmacokinetics and tumor delivery of siRNA polyplexes. A library of diblock polymers was synthesized, all containing the same pH-responsive, endosomolytic polyplex core-forming block but different corona blocks: 5 kDa (benchmark) and 20 kDa linear polyethylene glycol (PEG), 10 kDa and 20 kDa brush-like poly(oligo ethylene glycol), and 10 kDa and 20 kDa zwitterionic phosphorylcholine-based polymers (PMPC). *In vitro*, it was found that 20 kDa PEG and 20 kDa PMPC had the highest stability in the presence of salt or heparin and were the most effective at blocking protein adsorption. Following intravenous delivery, 20 kDa PEG and PMPC coronas both extended circulation half-lives 5-fold compared to 5 kDa PEG. However, in mouse orthotopic xenograft tumors, zwitterionic PMPC-based polyplexes showed highest *in vivo* luciferase silencing (>75% knockdown for 10 days with single IV 1 mg/kg dose) and 3-fold higher average tumor cell uptake than 5 kDa PEG polyplexes (20 kDa PEG polyplexes were only 2-fold higher than 5 kDa PEG). These results show that high molecular weight zwitterionic polyplex coronas significantly enhance siRNA polyplex pharmacokinetics without sacrificing polyplex uptake and bioactivity within tumors when compared to traditional PEG architectures.

Graphical abstract

Correspondence to: Craig L. Duvall.

Orcid: Taylor E. Kavanaugh: 0000-0002-8798-2670

Craig L. Duvall: 0000-0003-3979-0620

Associated Content: Supporting Information: The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.7b01110.

Notes: The authors declare no competing financial interest.

Keywords

siRNA polyplexes; zwitterionic; phosphorylcholine; pharmacokinetics; tumor delivery

There has been great interest in the development of small interfering RNAs (siRNAs) as human therapeutics for a variety of diseases, including cancer, with over 50 clinical trials completed or currently in progress.^{1,2} However, because of the poor pharmacokinetic properties of free siRNA, there remains an unmet need for carriers with optimized systemic bioavailability and delivery to solid tumors.³ Because tumors are perfused with a relatively small fraction of the body's blood volume, siRNA therapeutics must remain stable and inert in the systemic circulation for extended time in order to maximize the opportunity for passive accumulation within a tumor.⁴ The carrier must also be actively internalized and retained within the tumor cells rather than being transported back out of the tumor or being reabsorbed into the systemic circulation.

Upon intravenous administration, polyplexes encounter diverse delivery challenges that cause polyplex destabilization and/or removal by phagocytic cells, resulting in rapid clearance of the majority of the injected dose.^{5,6} Polyplexes can disassemble in circulation when they encounter serum proteins that penetrate polymer coronas or anionic heparan sulfates at the kidney glomerular basement membrane that compete with electrostatic interactions between polymer and siRNAs; free uncomplexed siRNA is then rapidly filtered for removal in the urine.^{6–8} Moreover, protein adsorption significantly affects biodistribution of polyplexes by marking them for recognition and phagocytosis by macrophages of the mononuclear phagocyte system (MPS) and/or potentially activating the complement pathway.^{5,9,10}

Polyplex surface chemistry is one of the most influential factors determining pharmacokinetics *in vivo* because physicochemical surface properties like charge and hydrophilicity dictate nature and level of adsorption or penetration by proteins and other molecules such as heparan sulfates.¹¹ The most common and exhaustively explored surface modification method for increasing particle stability, reducing protein adsorption, and improving pharmacokinetics is the functionalization of particles with a PEG corona (PEGylation). The importance of PEG molecular weight, architecture, and surface density for increasing particle circulation time has been widely studied.^{12–16} However, proteins can penetrate PEG layers, resulting in opsonization, destabilization, and MPS accumulation.^{8,17}

Additionally, many studies have shown that PEG can decrease overall target (*i.e.*, tumor) cell uptake once the carrier reaches the desired tissue.^{17,18}

A promising alternative to PEGylation is “zwitteration” of polyplex coronas. Zwitterionic surfaces are extremely hydrophilic because they are hydrated through strong electrostatic interactions, whereas PEGylated surfaces interact with water molecules through hydrogen bonding.¹⁹ Therefore, molecules that hydrate zwitterionic polymers are structured in the same way as in bulk water. This arrangement makes zwitterionic polymers thermodynamically unfavorable for protein adsorption, because there is no gain in free energy from displacing surface water molecules with protein.^{19,20} In general, zwitterionic coronas have been shown to improve *in vitro* stability, cell uptake, and pharmacokinetics of some nano-carriers relative to both PEGylated and unmodified carriers.^{21,22}

One type of zwitterion, phosphorylcholine, has found particularly widespread use for antifouling applications and is a component of FDA-approved contact lenses and drug-eluting stents.^{19,23,24} Phosphorylcholine-based polymers (*e.g.*, polymethacryloyloxyethylphosphorylcholine, PMPC) are hemo-compatible, easy to synthesize, and can mimic nonthrombogenic surfaces of red blood cells (RBCs), which contain many phosphorylcholine groups. In the context of nucleic acid delivery, Ukawa and colleagues have used PMPC coatings in GALA-modified lipid nanoparticles to increase their plasmid DNA transfection *in vitro*.²⁵ PMPC has also been recently applied for tumor delivery of siRNA *in vivo*. Yu and colleagues used PMPC-based cationic polymers to intravenously deliver siRNA against MDM2, reducing NSCLC tumor growth *in vivo* compared to scrambled controls, but there was no analysis of pharmacokinetics, no analysis of per cell particle uptake, and no comparison to PEGylated polyplexes.²⁶ There remains a need to comprehensively benchmark PMPC against traditional PEG architectures for *in vivo* pharmacokinetics, siRNA delivery, and activity within tumors.

Previous work in our lab focused on optimization of the polyplex core-forming block, resulting in the identification of a leading composition containing a balanced ratio of cationic and hydrophobic monomers (dimethylamino ethyl methacrylate (DMAEMA), and butyl methacrylate (BMA), respectively).²⁷ This optimization study solely utilized 5 kDa linear PEG as the corona-forming block. Here, we preserved the optimal core-forming DMAEMA-*co*-BMA composition and chain extended various corona-forming blocks in order to dissect the impact of corona chemistry on *in vivo* stability, pharmacokinetics, tumor accumulation, and tumor gene knockdown. We and others have sought to improve PEGylated nanocarrier pharmacokinetics through the use of a brush-like PEG architecture or high molecular weight Y-shaped PEGs, to varying degrees of success.^{14,15,28–30} In this study we compared PMPC coronas to these PEG architectures in addition to the linear 5 kDa PEG. We analyzed these polyplex surface materials using a number of techniques that quantify protein adsorption, polyplex stability, *in vitro* uptake and bioactivity, as well as *in vivo* pharmacokinetics and tumor gene silencing activity.

Results and Discussion

Synthesis of Diblock Copolymers with Varied Corona-Forming Polymer Blocks

Six diblock copolymers were synthesized with a pH-responsive block comprising a random copolymer of dimethylaminoethyl methacrylate (DMAEMA) and butyl methacrylate (BMA) at equimolar ratio and a total degree of polymerization of approximately 150. The polyplex corona-forming blocks consisted of 5 kDa linear PEG, 20 kDa linear Y-shaped PEG, 10 kDa poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA), 20 kDa POEGMA, 10 kDa zwitterionic PMPC, or 20 kDa zwitterionic PMPC corona (Figure 1). The 5 kDa linear PEG and 20 kDa linear Y-shaped PEGs were purchased, conjugated to the RAFT (reversible addition-fragmentation chain transfer) chain transfer agent, and then chain extended with RAFT to form the core-forming DMAEMA-co-BMA block. For the POEGMA and PMPC polymers, the core-forming DMAEMA-co-BMA block was first RAFT-polymerized and was subsequently extended using RAFT to polymerize two variants of each hydrophilic block composition near their target molecular weights of 10 kDa and 20 kDa. All diblock polymers were well-matched in terms of consistent DMAEMA-co-BMA block size and composition (approximately 150 degrees of polymerization with 50% of each monomer), and all polymers tested had relatively low polydispersity indices (PDI) ranging from 1.0 to 1.3 (Table 1, ¹H NMR methods detailed in Supporting Information Figures S1–S3). These properties were important to control because small changes in core length or composition could affect the polyplex performance independent of corona chemistry.²⁷ The 5k linear PEG, 10k PMPC, and 10k POEGMA corona lengths were chosen because they were the shortest corona lengths that would form relatively mono-disperse polyplex structures. The 20k PMPC and 20k POEGMA were chosen as standards to compare to the 20 kDa Y-shaped PEG, which has been used in FDA-approved drugs for extending circulation time.^{14,31}

Size and Stability Characterization of siRNA Polyplexes

Polyplexes were formed by first mixing polymer and siRNA at various $N^+:P^-$ ratios (number of protonated polymer amines:number of siRNA backbone phosphates) in pH 4.0 citrate buffer, and then the pH was raised to 7.4 (Figure 1). Based on a Ribogreen assay, all polyplexes reached an encapsulation efficiency of around 75–80% by $N^+:P^-$ 10, and slightly higher encapsulation efficiencies were achieved at $N^+:P^-$ 20 (Figure 2A). To determine the best $N^+:P^-$ ratio to use in subsequent testing of this library of polymers, we evaluated the average stability differences between polyplexes at $N^+:P^-$ 10 and $N^+:P^-$ 20 after a brief (30 min) incubation in 30% fetal bovine serum (FBS). By measuring the Forster resonance energy transfer (FRET) signal between co-encapsulated fluorescent siRNAs relative to the signal of polyplexes unchallenged by FBS, we observed a decrease in average stability of all polyplexes at $N^+:P^-$ 10 relative to $N^+:P^-$ 20. Average stability ranged from 75 to 86% FRET at $N^+:P^-$ 20 and from 42 to 48% FRET at $N^+:P^-$ 10 (Figure 2B). Because of these results, we selected the $N^+:P^-$ 20 ratio for all further studies. At this short serum incubation time, there were no significant differences between polyplexes of different coronas at a given $N^+:P^-$ ratio.

Importantly, despite their varied corona molecular weights and characteristics, all polyplexes had similar average hydro-dynamic diameters of approximately 100 nm and showed no

significant differences in surface charge (near neutral zeta potential) (Figures 2C–I and S4). Thus, although size and surface charge are known to affect pharmacokinetics, these factors were constant among each of the polyplexes despite different hydrophilic block chemistries.¹⁰ Polyplex size and stability evaluated under increasing salt concentrations showed that size of most polyplexes was only slightly affected by the addition of 0.1 M salt (Figure 2D–I). However, at 0.25 M NaCl, the 20k PMPC and 20k PEG polyplexes appeared most resistant to destabilization by increasing salt concentrations, while the 5k PEG and POEGMA corona polyplexes lost their uniform size distribution. This result suggests that larger coronas improved stability for linear PEG and zwitterionic PMPC coronas, but in the case of POEGMA-based polyplexes, increasing corona size did not improve stability. Polyplexes made with the longer 20k POEGMA corona, in addition to being less stable than other polyplexes, were also more polydispersed at baseline in low-salt conditions compared to other polyplexes. This is possibly due to excessive bulkiness, making it more difficult for this polymer to form tightly packaged micelles through electrostatic interactions in the core.³² While these POEGMA polymers were selected because the 950 Da side chains form more hydrophilic blocks than 300 Da OEGMAs, high molecular weight monomers are not as well studied as shorter OEGMA monomers, and their extended side chains may cause considerable steric repulsion between corona-forming blocks, a potentially destabilizing factor.^{29,33}

In order to maximize polyplex accumulation at the site of the tumor, polyplexes must resist destabilization in circulation.⁴ The main sources of polyplex instability in the blood circulation include serum and anionic heparan sulfates in the kidney glomerular basement membrane, the latter of which can interact with positively charged components of siRNA polyplexes and result in decomplexation.^{5–7} To determine the impact of particle surface chemistry on polyplex stability, we challenged polyplexes containing FRET pair-loaded siRNA with serum or heparin salts. We found that at higher serum levels (30%), alternative corona chemistries improved stability compared to 5k linear PEG coronas, but there were few stability differences between individual coronas (Figure S5). At 10% serum, all polyplexes at $N^+:P^-$ 20 resisted destabilization regardless of corona block (Figure S5). As shown previously in Figure 2B, polyplex $N^+:P^-$ ratio appeared more important for short-term serum stability than corona block differences.

Heparin salt-induced destabilization showed a greater dependency on corona composition. In heparin salts at a range of concentrations (Figure 3A–C), 20k PEG and zwitterionic 20k PMPC coronas provided the greatest stability over time compared to all other polyplex coronas. In 100 U/mL heparin over 100 min, the average FRET signal for 20k PMPC and 20k PEG samples was significantly higher than that of 5k PEG, 20k POEGMA, and 10k PMPC ($p < 0.05$), indicating greater resistance to charge-induced destabilization. The 20k PMPC and 20k PEG also performed best with 60 U/mL heparin, only decreasing in average FRET signal by 40 and 36%, respectively, while the FRET signal in all other polyplex samples decreased by 56–63%. At each heparin condition, 10k POEGMA was intermediately stable, and this was most apparent at 20 U/mL heparin, when 10k POEGMA did not diverge from 20k PEG and 20k PMPC until roughly 55 min of incubation. The other polyplexes, 5k PEG, 10k PMPC, and 20k POEGMA, were consistently the least heparin stable. While larger corona molecular weight improved stability for linear PEGylated and

zwitterated polyplexes, this advantage did not hold true for POEGMA coronas. This could possibly be due to unfavorable steric properties of the bulky POEGMA side chains, which could potentially reduce core stability at baseline.³² While some heparin may be a binding free, uncomplexed polymer, the results of our Ribogreen assay (Figure 2A) indicate that the packaging of siRNA is the same for all polyplexes at $N^+:P^-$ 20, so the molar amount of free polymer is consistent between all polyplexes and unlikely to influence these comparisons. Heparin binding free polymer is also unlikely based on the polyplex DLS traces (Figure 2D–I) that do not show evidence of free polymer and because any uncomplexed polymer in solution is micellar (due to the presence of hydrophobic BMA in the core-forming block), thus making DMAEMA unavailable for binding.²⁷

***In Vitro* Characterization**

In order for the polyplexes to enable siRNA bioavailability in the target cell cytoplasm, the polyplexes must exhibit efficient cell uptake, pH-responsive endosomal escape, and target gene knockdown, while also not being cytotoxic to normal (noncancerous) cells. The core-forming block of each polyplex in our library, consisting of DMAEMA-*co*-BMA, has previously been optimized for pH-responsive endosomolytic behavior, and we hypothesized that the various corona chemistries would not affect this property.²⁷ We measured pH-dependent membrane disruptive behavior, a surrogate assay for endosome escape capability in which polyplex samples are incubated with RBCs in buffers of progressively lower pH that mimic extracellular, early/late endosome, and lysosome environments.³⁴ All of the polyplexes produced membrane disruptive activity at pH values at or below 6.8, corresponding to pHs found in the endolysosomal pathway, but no hemolytic activity occurred at physiological and extracellular pH of 7.4 (Figure 4A). Because the pH-responsive behavior of these polyplexes is controlled by their core blocks, it was expected that the different coronas would not differentially impact endosomolytic behavior. These trends were independent of polyplex concentration in the range tested (Figure S6).

We next screened for toxicity of all polyplexes in luciferase-expressing NIH3T3 fibroblasts (Figure 4B), in order to determine whether the polyplexes were harmful to normal and noncancerous cells. At 48 h post-treatment, none of the polyplexes significantly affected viability levels relative to untreated cells, with the exception of 10k PMPC. Average viability of 10k PMPC was still quite high, at 87%, indicating it was also relatively nontoxic. We also evaluated cytotoxicity of all polyplexes bearing scrambled siRNA in MDA-MB-231 cancer cells. All cell viability values were still >75% after 48 h incubation with high polyplex concentrations (Figure S7).

We next evaluated uptake of polyplexes by MDA-MB-231 breast cancer cells (Figure 4C). Overall, polyplexes made with smaller molecular weight corona blocks had significantly higher mean fluorescence intensities compared to more shielded polyplexes comprising longer corona-forming blocks ($p < 0.001$, $n = 3$). In comparing different polyplex corona-forming block chemistries, the mean fluorescence intensity of 20k PMPC was >20k PEG polyplexes, and 10k PMPC had the highest uptake levels overall ($p < 0.001$, $n = 3$). For *in vitro* uptake, lower molecular weight coronas are often associated with increased cell uptake, because the cationic PDMAEMA components are less shielded.³⁵ However, polyplexes

made with shorter corona-forming blocks are generally less stable and therefore likely to produce less favorable *in vivo* pharmacokinetic properties. We also stratified polyplex uptake based on the percent of cells found within low, medium, and high uptake subsets (Figures 4D and S8a) in order to further interrogate differences in the distribution of polyplex uptake levels within the different treated cell populations. The 20k PEG had the highest percent of polyplex uptake positive cells in the low uptake group by a significant margin ($p < 0.001$), with 47% of cells in this group, while all other polyplex types ranged from 31 to 36% of cells in the low uptake group. In comparison, 10k PMPC had the highest percent of cells in the high group, at 31%. While more shielded than both 5k PEG and 10k POEGMA, 20k PMPC had a similar percent of cells in the “high” uptake category (14–17%) as the shorter corona polyplexes. In the high uptake category, 20k PEG polyplexes had the lowest percent of cells (8.9%), after 20k POEGMA (1.22%), which was significantly lower than the percent of cells treated with 20k PMPC polyplexes in the high category (8.9% vs 15%, $p < 0.04$). These combined data provide support that PMPC-based coronas have higher cell uptake properties compared to their PEGylated counterparts, suggesting that zwitterionic coronas provide polyplexes with stealth properties without limiting polyplex uptake as significantly as incorporation of high molecular weight PEG coronas. These data suggest that PMPC is less susceptible to the well-described “PEG dilemma” in drug delivery.⁸ Histograms of these cell populations can be found in Figure S8b.

After confirming pH-responsiveness, cytocompatibility, and tumor cell uptake behavior of the polyplexes, we evaluated knockdown of the model gene luciferase in luciferase-expressing MDA-MB-231 breast cancer cells (Figure 4E), across a range of doses. At the highest dose of 100 nM siRNA after 48 h, cells exposed to 5k PEG, 20k PEG, or either PMPC corona all retained <10% luciferase activity (90% knockdown), which was significantly lower than 10k POEGMA, with 50% knockdown, and 20k POEGMA, with no significant knockdown. At 75 nM, differences between linear PEG and PMPC polyplexes emerged. The 10k PMPC corona polyplexes had the best bioactivity, with 8% remaining luciferase activity, followed by 20k PMPC and 5k PEG, which were not significantly different from each other at 14% luciferase activity. The 20k PEG coronas at this dose had significantly higher remaining luciferase activity than 20k PMPC and 5k PEG (29%), providing evidence that PMPC-based coronas have increased bioactivity compared to their linear PEG counterparts. These general trends are also evidenced by the IC_{50} values for each polyplex (Figure 4F). Polyplexes containing 10k PMPC had the lowest IC_{50} , at 18 nM, compared to 5k PEG at 49 nM. The 20k PMPC-based polyplexes also had a significantly lower IC_{50} than 20k PEG polyplexes (55 nM vs 64 nM), with values similar to the IC_{50} of less well-shielded 5k PEG. All polyplex IC_{50} averages were significantly different from each other ($p < 0.04$). In general, the POEGMA-based coronas did not produce desirable levels of *in vitro* bioactivity, while all other polyplex formulations produced potent knockdown with negligible cytotoxicity.

Protein Adsorption and Complement Activation

Both PEGylation and zwitteration are designed to reduce protein adsorption to nanocarriers because opsonization makes nano-carriers more identifiable to macrophages of the MPS and/or destabilizes polyplexes. If complement protein C3b adsorbs, the complement cascade

can be activated, further recruiting immune cells and promoting rapid clearance.⁹ We used isothermal titration calorimetry (ITC) and a hemolysis-based complement assay to evaluate how PEGylation and zwitteration differentially affect protein adsorption as a function of block molecular weight.

In ITC, a protein solution is slowly titrated into a solution of polyplexes, and changes in heat resulting from their interaction are recorded (Figure 5A). These heat changes can be used to calculate entropy, enthalpy, and Gibb's free energy of interaction between the protein and nanocarrier.^{36,37} Albumin was used here as a model for serum proteins, since it comprises the largest percent of human serum compared to other proteins and is known to opsonize PEGylated nanoparticles.^{8,38} Overall, each polyplex, whether PEGylated or zwitterated, had a positive Gibb's free energy of interaction with albumin (Figure 5B). This indicates that albumin binding was not spontaneous and therefore not favored. However, the magnitude of average ΔG values was increased for the higher molecular weight coronas as compared to their lower molecular weight counterparts, indicating albumin adsorption was least favorable for these polymers. The 20k linear Y-shaped PEG corona had the largest ΔG values of any polyplex, followed by the 20k PMPC, and both were significantly higher than ΔG of 5k PEG ($p < 0.05$, $n = 3$). The significant differences between polyplex coronas of higher and lower molecular weight indicate the importance of corona molecular weight in blocking protein adsorption.

All polyplexes with neutrally charged, hydrophilic coronas were compared to a positive control polyplex containing 100% cationic PDMAEMA in its corona, which is known to significantly adsorb proteins at physiological pH.^{14,39,40} As expected, this cationic control showed negative ΔG values averaging -35 kcal/mol, indicating a highly favorable interaction with albumin. While all raw ITC data showed endothermic heat changes for all of the shielded polyplexes, the cationic control polyplexes had highly exothermic heat changes (Figure S9). The stark contrast between the cationic control polyplex and the neutral polyplex coronas demonstrates the overall impact of a “stealth” corona in reducing protein adsorption. Because albumin is not the only component of a nanoparticle protein corona, this contrast was also demonstrated for whole FBS, using a qualitative agarose gel binding assay (Figure S10).³⁸ The cationic PDMAEMA-corona particles retained a high FBS signal in the loading well, where polyplexes are known to remain (instead of migrating through the gel), while the protein signal from wells containing all other polyplexes was much lower and closely matched the signal from wells containing only FBS.²⁷ This implies that overall serum adsorption is low for all shielded polyplexes with the various coronas, but high for cationic PDMAEMA particles.

In order to evaluate potential complement protein adsorption by the various corona chemistries, we used a hemolytic assay modified from Bartlett and colleagues.⁴¹ Polyplexes were incubated with various dilutions of human complement sera, and then antibody-sensitized sheep erythrocytes were added to each mixture. If complement proteins do not adsorb to polyplexes, then they are available in solution to lyse the erythrocytes. However, if adsorption does occur, RBC lysis is reduced as fewer complement proteins are available to cause lysis (Figure 5C). For all polyplexes, we did not observe significant differences in lysis compared to the complement only protein controls at all concentrations of complement

sera, meaning that complement adsorption was negligible (Figure 5D). The unshielded cationic polyplexes with a PDMAEMA corona, on the other hand, robustly reduced lysis compared to the complement only control, indicating significant adsorption, as expected. The 20k PEOGMA corona polyplex exhibited slightly elevated lysis levels compared to the protein only control, probably as a result of its greater instability in serum, but overall, the coronas tested do not react spontaneously with complement proteins to any significant degree, in agreement with the results for albumin adsorption as measured by ITC. These data also indicate that the complement assay may not be as sensitive as the ITC method at detecting small functional differences in protein adsorption.

***In Vivo* Pharmacokinetics**

We next studied the *in vivo* pharmacokinetics of the polyplex library after intravenous administration. Traditional methods of characterizing nano-carrier pharmacokinetics rely on multiple blood draws and extrapolation to determine initial nanocarrier blood concentrations. Intravital confocal laser scanning microscopy (IVM), on the other hand, provides real time, continuous tracking of fluorescence in the mouse ear blood vessels and requires fewer animals.⁴² Recently intravital microscopy has been used to monitor polyplexes in blood circulation, particularly to understand the impact of core stabilizing components on circulation time and to characterize the impact of species-specific immune state on nanoparticle clearance.^{43–45} IVM provides a more absolute quantification of particle pharmacokinetic parameters and is therefore a more robust way to discern differences between PEGylated and zwitterionic coronas. For our study, polyplexes were loaded with Cy5-conjugated cargo, and the fluorescence signal tracked for 20 min after injection.

Intravital microscopy studies revealed improved pharmacokinetic properties for 20k PMPC and 20k PEG, in agreement with our *in vitro* stability results, as visualized in Figure 6A. Pharmacokinetic curves of blood circulation (Figure 6B) were fit to the data, and area under the curve values for 20k PMPC and 20k PEG were 586 and 507 (mg-min)/(L) respectively, roughly three to four times higher than all other polyplexes tested (Figure 6C). Average circulation half-lives for 20k PMPC and 20k PEG were 26 and 22 min, respectively, while circulation half-lives for all other polyplexes ranged from 5 to 8 min (Table 2). Average circulation half-life for free nucleic acid was <2 min. Similarly, 20k PMPC and 20k PEG had much lower clearance values than any other polyplexes studied (Table 2). Organ biodistribution (Figure S11) studies at 20 min revealed that for all of the polyplexes, the greatest percentage of the fluorescent siRNA was localized in the kidneys, followed by the MPS organs (liver and spleen). For all polyplexes, <50% of the total fluorescence was localized in the kidney, which is an improvement over many other polyplex systems in the literature which are more rapidly disassembled in the kidney and therefore have higher kidney accumulation.^{45–49} However, this is likely due in large part to the balance of cationic and hydrophobic monomers in the core-forming block of these polyplexes, which reduces heparan sulfate-based disassembly as our group has shown previously.²⁷

The pharmacokinetic properties of 20k PMPC and 20k PEG tested here are field-leading relative to other published polyplex siRNA delivery systems. For example, the cyclodextrin-based polyplex system, CALAA-01, that was administered to humans in the first ever RNAi

clinical trial, cleared from humans, monkeys, mice, and rats below the detection limit only 30 min after intravenous injection.⁵⁰ Our 20k PEG and 20k PMPC polyplexes, on the other hand, have only just reached their half-life after approximately 30 min. Others have sought to improve polyplex circulation stability through the addition of cholesterol-modified siRNA in combination with 20 kDa PEG-conjugated cationic polymers, but such modifications only increased half-lives to around 6–10 min.⁴⁴ An additional approach to increasing circulation time in a similar system is polyplex core cross-linking, but this only increased circulation half-lives to roughly 10 min based on intravital microscopy.⁴⁶ Our polyplexes effectively combine a highly stabilized core with highly stabilizing, protein-stealth coronas, increasing their circulation times beyond the gold standards in the field.

In all, our results indicate that polyplexes of 20k PMPC and 20k PEG were the most blood stable and may also be most resistant to natural clearance mechanisms like renal heparan sulfate-mediated clearance, protein adsorption, and phagocytosis. These properties suggest they are the leading candidates for development for oncological siRNA therapeutics. Similar to our *in vitro* results, the high molecular weight POEGMA coronas did not exhibit the same beneficial properties, most likely due to the poor stability properties of these polyplexes.

***In Vivo* Tumor Gene Silencing and Biodistribution**

When nanoparticles circulate longer in the bloodstream, their systemic bioavailability increases, and they have a greater opportunity to accumulate within tumors. Because our higher molecular weight coronas had such dramatic improvement in circulation half-lives and clearance properties, we hypothesized that they would also improve tumor accumulation and gene silencing. Therefore, we selectively compared the ability of 20k PEG and 20k PMPC to achieve tumor cell delivery and target gene silencing of the model gene luciferase in a mouse orthotopic model of breast cancer. These leading formulations were benchmarked against our previous gold standard 5k PEG polyplexes.^{27,51}

Mice bearing luciferase-expressing MDA-MB-231 mammary fat pad tumors were intravenously injected with 20k PMPC, 20k PEG, or 5k PEG polyplexes bearing 1 mg/kg antiluciferase or scrambled control siRNAs. Each animal received only one treatment, and tumor luminescence was monitored for a 10 day period post-injection. We compared the relative luminescence of each individual tumor to its luminescence prior to polyplex injection, and the luminescence values for the luciferase siRNA polyplex-treated tumors were compared to average luminescence values for the tumors from scrambled control siRNA polyplex-treated mice. There were no significant differences in relative luminescence between any scrambled polyplex group throughout the study period.

Throughout the 10 day period post-injection, mice treated with zwitterionic 20k PMPC polyplexes containing luciferase siRNA exhibited more potent and long-lasting luciferase silencing than either PEG-based polyplex (Figure 7A), with significantly increased knockdown on days 3–7. Throughout the treatment, relative luminescence values for 20k PMPC averaged about 20% that of scrambled controls, indicating 80% knockdown. The differences between 20k PEG and 5k PEG were not significant, but average knockdown potency tended to be slightly higher for the 20k PEG than 5k PEG polyplexes. Average knockdown of luciferase by 20k PEG ranged from 75% on day 1 to 36% on day 10

compared to scrambled polyplex controls, and knockdown of luciferase by 5k PEG ranged from average 56% on day 1 to 35% on day 10. This study suggests that, despite their similar pharmacokinetic properties, 20k PMPC has higher *in vivo* bioactivity compared to 20k PEG (representative tumor luminescence images demonstrating these trends are displayed in Figure 7B). Mouse body weight was recorded each day of the study period as an indicator of toxicity, and there were no significant differences between any polyplex treatment group and untreated tumor-bearing mice (Figure S12).

In order to elucidate the mechanism behind the increased tumor gene knockdown of zwitterionic 20k PMPC, we also studied the *in vivo* biodistribution and tumor cell uptake of zwitterionic vs PEGylated polyplexes. We injected tumor-bearing mice with 20k PMPC, 20k PEG, and 5k PEG polyplexes bearing Cy5-labeled cargo. After 24 h, the tumors were removed and dissociated into a single cell suspension to measure polyplex internalization level per tumor cell (tumor cells identified as GFP positive based on reporter transduced prior to tumor cell inoculation into mice). After 24 h of accumulation time, zwitterionic 20k PMPC showed significantly higher tumor cell uptake levels than either 5k or 20k PEGylated polyplexes (Figures 7C,D). Mean Cy5 fluorescence intensity in GFP positive (tumor) cells for 20k PMPC was 3-fold higher than that of 5k PEG and almost 2-fold higher than 20k PEG. Additionally, 20k PMPC had the highest percentage of Cy5-positive tumor cells, with roughly 90% positive, while the average percent uptake for 5k PEG and 20k PEG was 40% and 80%, respectively, at 24 h (Figure 7E). These data suggest that the longer half-lives of 20k PMPC and 20k PEG played an important role in their tumor uptake and that 20k PMPC coronas do not sacrifice cell uptake as much as PEG-based coronas. Organ biodistribution data based on overall fluorescence at this 24 h time point revealed that average radiance in tumors was 1.2–1.9-fold higher than liver average radiance and 2.4–7.4-fold higher than heart and lung average radiance for all of the polyplexes studied (Figure S13).

The increased *in vivo* uptake of 20k PMPC polyplexes compared to 20k PEG polyplexes indicates that the higher levels of gene knockdown of 20k PMPC are driven by a combination of increased circulation time and higher rate of cell uptake of PMPC-based polyplexes. To further elucidate the latter mechanism, we compared the *in vitro* uptake properties of 5k PEG, 20k PEG, and 20k PMPC over a 2 day time course (Figure 7f). Measurements at early time points of 30 min and 4 h showed that 20k PMPC was taken up by MDA-MB-231s more rapidly than 20k PEG, with 2-fold higher uptake at each time point ($p < 0.002$, $n = 3$). 20k PMPC uptake was also significantly higher than 20k PEG corona polyplexes at 24 h. 5k PEG polyplexes had similar uptake levels to 20k PMPC at earlier time points *in vitro*. However, 5k PEG polyplexes lack the same level of corona shielding, resulting in higher uptake levels *in vitro*, but less stability in circulation and therefore reduced *in vivo* tumor biodistribution. Additionally, 20k PMPC more rapidly penetrated the cell population homogeneously relative to either PEGylated corona. At 4 h, 5k PEG showed 60% positive cells compared to 95% for 20k PMPC polyplexes ($p < 0.01$, Figure S14). By 48 h, 20k PMPC polyplexes exhibit significantly increased uptake compared to both 5k PEG and 20k PEG coronas ($p < 0.0003$). Thus, 20k PMPC polyplexes maximize both serum stability and increased cell uptake. While less-shielded 5k PEG polyplexes have a higher uptake at early time points, more serum-stable 20k PEG polyplexes catch up to 5k PEG polyplexes over time. On the other hand, 20k PMPC combines both a high rate of uptake

and the increased stabilization properties necessary for continued uptake over longer periods of time in the presence of serum, enabling its improved *in vivo* tumor uptake compared to PEGylated polyplexes.

In order to dissect whether the *in vivo* relative tumor cell uptake differences (Figure 7D) were driven in part by differences in tumor tissue penetration of the polyplexes, we investigated penetration in an *ex vivo* model, using MDA-MB-231 tumors excised from nude mice. After 24 h of polyplex incubation with tumors, all polyplexes penetrated to similar depths from the tumor edge (Figure 7G). Quantification based on line profiles drawn from the tumor edge indicated that, particularly close to the tumor edge (10–20 μm), average intensities of 20k PMPC polyplexes were significantly higher ($p < 0.01$) than 5k PEG and 20k PEG polyplexes, at 1.5-fold and 2.1-fold higher, respectively. Overall area under the average intensity curve was also highest for 20k PMPC. However, overall distribution of polyplexes based on depth was similar, as seen in representative histology images (Figure 7h). While the highest polyplex signal intensities were observed within 40 μm from the tumor edge, the average signal from all polyplex-treated tumors was still higher than the signal from untreated tumors deeper in the tumor (40–100 μm), with no significant differences between individual polyplex types at these deeper tumor regions.

The 5k PEG polyplex intensities near the tumor edge are higher than the 20k PEG intensities in this study, which is different than the tumor delivery levels seen *in vivo*, due to the *ex vivo* nature of the experiment; these polyplexes did not require stability in circulation prior to reaching the tumors and, with lower molecular weight PEG, are less shielded to begin with, allowing for increased tumor uptake. Given this result, it is noteworthy that the more well-shielded 20k PMPC-based polyplexes displayed higher average intensities at low penetration depths than 5k PEG polyplexes, despite their higher molecular weight coronas, further supporting the improved uptake properties of PMPC-corona polyplexes.

The similar depth penetration profile of each of these polyplexes indicates that the higher bioactivity of 20k PMPC corona polyplexes is driven primarily by higher uptake properties and not by differences in tissue penetration. These penetration profiles were further corroborated with histological evaluation of tumors after *in vivo* tail vein administration of Cy5-bearing polyplexes (Figure 7H). Here again, Cy5 signal from each polyplex could be found deep within tumor sections and relatively well-penetrated. While all polyplexes appear to have a similar histological distribution in the tumor, the overall amount of uptake is different depending on polyplex type, as we quantified comprehensively through *in vivo* flow cytometry.

Our combined tumor gene knockdown and tumor uptake data indicate that high molecular weight zwitterionic PMPC coronas perform significantly better than their linear PEG counterparts. Our work thoroughly demonstrates the *in vivo* advantages of PMPC compared to PEG in systemically delivered siRNA polyplexes, and other studies in nonpolyplex systems support and corroborate our findings. In gold nanoparticles, for example, multiple studies have shown that zwitterionic coatings increase accumulation at the site of a tumor over PEGylated surface coatings.^{52,53} Similarly, in protein-based nanoparticles, PMPC coatings improve tumor uptake over PEG-based copolymers, and this effect was also

primarily observed at later time points (12 h post-injection), implying better tumor accumulation and retention over time as our study reveals.⁵⁴

The observation that PMPC-based polyplexes improve cell uptake while reducing protein adsorption is somewhat counterintuitive and requires further mechanistic investigation, in both normal and cancer cells. One potential explanation is that zwitterionic phosphorylcholine particle surface chemistries improve tumor accumulation because they promote association with cell membranes, given their similarity to membrane phospholipid heads.⁵⁴⁻⁵⁷ It has also been observed that PMPC surface chemistries are particularly conducive to internalization by cancer cells, even more so than in normal cells.^{55,58} This may be due to the fact that many invasive and rapidly proliferating cancer cells, including the MDA-MB-231 cells in our study, have increased choline transport, due to their higher membrane synthesis and turnover.⁵⁹ All of these existing data are consistent with our own results comprehensively demonstrating increased tumor cell uptake of PMPC-based siRNA polyplexes compared to PEGylated polyplexes both *in vitro* and *in vivo*.

In comparing to other siRNA delivery work, our 20k PMPC polyplexes generated higher tumor gene knockdown compared to the few previous examples of *in vivo* PMPC-based siRNA delivery. In one study, siRNA-bearing PMPC-PDPA (poly-(diisopropanolamine ethyl methacrylate)) diblock copolymers achieved significant luciferase knockdown (up to 75%), but this level was achieved after six repeated injections of low polyplex doses (as opposed to our single 1 mg/kg dose), and these polyplexes were not compared to PEGylated counterparts.²⁶ Most top-performing polyplex systems are PEGylated and frequently suffer from low tumor uptake without addition of targeting ligands or cholesteryl stabilizing moieties.^{44,60} Our PMPC-based polyplexes, on the other hand, without active targeting, increased average tumor cell uptake 3-fold compared to 5k PEG and 2-fold compared to 20k PEG and achieved up to 90% Cy5-positive tumor cells after only a single, relatively low-dose administration. The 20k PMPC polyplexes preserve the stability advantages of high molecular weight stealth coronas while also producing higher cell uptake than high molecular weight PEGylated polyplexes.

These results are predicated on the assumed presence of the enhanced permeability and retention (EPR) effect in tumors, which enables nanoparticle extravasation through porous tumor vasculature. This effect has been subject to debate in recent literature, with most studies suggesting that this phenomenon is heterogeneous in human cancers.^{61,62} However, a recent clinical trial in human subjects directly confirmed the presence of the EPR effect in all of the patients studied (across multiple solid tumor types), showing micellar accumulation in tumor tissue but not in surrounding tissues.⁶³ As is the case for clinical, standard of care cancer therapeutics, it is true that some patients may benefit more than others from nanomedicines taking advantage of the EPR effect. Recent work has suggested that magnetic nanoparticles can be used as an imaging agent to predict whether EPR-based nanomedicines will be advantageous in different patients.⁶² These opportunities for personalized nanomedicine-based cancer treatment only intensify the need for improved polyplex stability and tumor accumulation in order to maximize the full potential of EPR across a varied patient population. In our study, we have shown that these goals are

achievable for siRNA polyplexes using zwitterionic PMPC surface chemistries more so than PEGylated ones.

Conclusion

Taken together, our data show that while high molecular weight PEG and PMPC coronas generally improve polyplex circulation time, zwitterionic PMPC coronas significantly improve *in vivo* tumor cell uptake and bioactivity compared to canonical PEGs. *In vitro*, larger molecular weight hydrophilic corona-forming blocks performed better in terms of increasing heparin stability and blocking protein adsorption. In these and other assays, POEGMA was the exception to this trend, and the PEG brushlike architecture as a rule did not perform as well as PMPC and linear PEG corona-forming blocks in the polyplex format used in these studies. These comprehensive *in vitro* data corroborated our *in vivo* pharmacokinetic data, which used intravital microscopy for elucidating the intravenous impact of polyplex corona chemistry. This method quantitatively showed that both high molecular weight corona-forming blocks comprising either 20k Y-shaped PEG or 20k zwitterionic PMPC improve polyplex circulation half-lives over shorter coronas. These results motivated more in-depth studies of the 20k PEG and PMPC formulations in comparison to our 5k PEG benchmark. Our *in vivo* tumor uptake and gene knockdown studies show that high molecular weight zwitterionic coronas significantly improve *in vivo* gene knockdown and tumor cell uptake compared to high molecular weight PEGylated polyplexes. This work has important implications for the optimization of siRNA nanocarriers used for systemic cancer therapeutics. PEG has long been regarded as the gold standard, while increasingly well-characterized noncanonical surface chemistries remain under utilized. We have shown that PMPC, a biocompatible material that is a component of FDA-approved products, is easily and controllably polymerized and significantly improves tumor cell uptake and knockdown activity of siRNA polyplexes over PEG-based structures, encouraging further development of zwitterionic surface chemistries for siRNA oncological therapeutics.

Materials and Methods

Materials

All materials were purchased from Sigma-Aldrich unless otherwise described. Inhibitors were removed from dimethylaminoethyl methacrylate (DMAEMA) and butyl methacrylate (BMA) using an activated aluminum oxide column. All DNA or siRNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA), and sequences can be found in our previous work.²⁷ In FRET experiments, *in vitro* cell uptake experiments, *in vivo* biodistribution, and pharmacokinetic experiments, DNA oligonucleotides were used as a model for siRNA oligonucleotides of the same length.

Polymer Synthesis

All polymers were synthesized using 4-cyano-4-(ethylsulfanylthiocarbonyl)sulfanylpentanoic acid (ECT) as an initial chain transfer agent. ECT was synthesized in house according to previously published methods.²⁷ 5k PEG ECT

was synthesized as previously described by coupling a 5 kDa hydroxyl-terminated PEG (JenKem, USA) to ECT by DCC DMAP coupling (Figure S1). For the coupling reaction, ECT was added to a reaction vessel at 10:1 mol equiv of 5 kDa or 20 kDa PEG (JenKem, USA) and dissolved in dichloromethane at 10 wt %/v. Dicyclohexyl carbodiimide (DCC) was then added to activate the carboxylic acids on ECT at a 1:1 molar ratio with ECT. After stirring 5 min, hydroxyl-terminated 5 kDa or 20 kDa PEG was added to the reaction mixture, followed by 4-dimethylaminopyridine (DMAP). DMAP was added at a 5:1 mol equiv to PEG. ECT was added to the 5 kDa PEG at a 10:1 molar ratio. Dicyclohexyl carbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were added at 5 mol equiv the amount of PEG. The coupling reaction was stirred at room temperature for 48 h, and the final product was purified as previously described.²⁷ From the 5 kDa or 20 kDa PEG macroCTA, DMAEMA and BMA were RAFT polymerized at 50:50 molar ratios using AIBN as an initiator (10:1 CTA:Initiator ratio) in 10% w/v dioxane. Reactions were planned with a degree of polymerization of 240, in order to achieve 75–80 repeating units each of DMAEMA and BMA (at a 65–70% monomer conversion rate). The reaction was nitrogen purged for 30 min and then was stirred at 65 °C for 24 h. The final reaction mixture was dialyzed into methanol for 2 days, then water for 2 days, and lyophilized. 20k PEG was synthesized using the same methods as for the 5k PEG polymers, but a 20 kDa Y-shaped hydroxyl PEG was conjugated to ECT to create the appropriate macroCTA. Zwitterionic PMPC was synthesized in a two-step process. First, DMAEMA and BMA were RAFT polymerized at the same monomer feed ratios, and conversion estimates are described above. ¹H NMR was used to evaluate the conversion rate (Figure S2). This random DMAEMA-BMA copolymer (DB ECT) was then used as a macroCTA to polymerize a homopolymer block of 2-(methacryloyloxyethyl) phosphorylcholine. For a 20 kDa corona or a 10 kDa corona, target degree of polymerizations of 75 and 40 were used, respectively. These polymerizations used AIBN at a 5:1 CTA:initiator ratio and were done at 10% w/v in anhydrous methanol solvent. Reactions were purged with nitrogen for 30 min before heating to 65 °C for 24 h. Final reaction products were dialyzed in methanol for 2 days, then water for 2 days, and lyophilized. Polymers with poly(ethylene glycol) ethyl ether methacrylate (POEGMA) ($M_n = 950$) in the corona were synthesized in a similar method using a 10:1 CTA:initiator ratio and dioxane as a reaction solvent (10 wt %/v). Prior to polymerization, inhibitors were removed from POEGMA monomers by first dissolving in anhydrous THF, running through an alumina column, and then drying using a rotary evaporator. For 10k POEGMA coronas and 20k POEGMA coronas, aimed degrees of polymerization were 19 and 31, respectively, with conversion rates of 60% and 65%, respectively.

All polymers were characterized using ¹H nuclear magnetic resonance spectroscopy (Bruker, 400 MHz). Polymer polydispersity was evaluated with DMF mobile phase gel permeation chromatography (GPC, Agilent Technologies, CA). For this measurement, all PEG or POEGMA polymers were dissolved in DMF containing 0.1 M lithium bromide at a concentration of 10 mg/mL. All NMR spectra are shown in Figure S3.

Polyplex Formation and Encapsulation Efficiency

All polyplexes used *in vitro* for this work were first complexed with siRNA in 10 mM citrate buffer at pH 4 for 30 min, followed by raising the pH to 7.4 using 10 mM pH 8 phosphate

buffer at 5× the volume of the pH 4 solution. Polyplex N⁺:P⁻ ratios were determined as the mole ratio of protonated amines from DMAEMA monomers (50% are assumed protonated at physiological pH) to the number of phosphate groups on the siRNA according to the following equation:^{27,64}

$$\text{nmol polymer} = \frac{(\text{nmol nucleic acid})(\text{base pairs nucleic acid})(2)(\text{N:P ratio})}{(\text{DMAEMA repeat units})(0.5)}$$

Unless otherwise noted, all *in vitro* polyplex characterization assays were performed at a dose of 100 nM siRNA and 2.7 μM polymer (N⁺:P⁻ 20), which corresponds to 0.074, 0.12, 0.093, 0.12, 0.093, and 0.12 mg/mL for 5k PEG, 20k PEG, 10k PMPC, 20k PMPC, 10k POEGMA, and 20k POEGMA, respectively.

Polyplex encapsulation efficiency at various N⁺:P⁻ ratios was evaluated using a Quant-iT Ribogreen assay kit (ThermoFisher, USA). Assay solutions were prepared based on manufacturer's instructions, and Ribogreen fluorescence was measured using the high range assay. Polyplex solutions were prepared at 100 nM siRNA, and 50 μL of polyplex solution was diluted by half in 1× TE buffer, followed by addition of 100 μL Ribogreen reagent to each well. Fluorescence was measured at 520 nm, and encapsulation efficiency was calculated by normalizing fluorescence of polyplex solutions to fluorescence of siRNA-only control solutions.

Polyplexes were prepared for TEM at 0.5 mg/mL polymer. Five μL polyplex suspensions were added to pure carbon TEM grids (TED Pella Inc., Redding, CA, USA) and blotted dry after 60 s. TEM grids were then counterstained with 3% uranyl acetate (5 μL) for 20 s and then dried overnight under vacuum. Images were obtained with a FEI Tecnai Osiris microscope (200 kV).

Polyplex Stability Evaluations

Polyplex diameters and zeta potentials were measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Westborough, MA). For these measurements, polyplexes were prepared at final concentrations of 0.167 mg/mL. For stability measurements, each polyplex solution was incubated with 0.1 or 0.25 M NaCl solutions.

Polyplex stability was also measured in FBS and heparan sulfate through a FRET assay described previously.^{14,27,51} Briefly, polyplexes were co-loaded with nucleic acids conjugated with either Alexa Fluor-488 or Alexa Fluor-546 dyes, forming FRET pairs. Intensity of fluorescence emission after excitation at 488 nm was measured at 514 and 572 nm using a fluorescence plate reader (Tea Infinite F500, Mannedorf, Switzerland). Percent FRET for each polyplex sample was calculated as

$$\frac{I_{572}}{I_{572} + I_{514}} \times 100$$

For the FBS-based FRET challenge, polyplexes were incubated at concentrations of 100 nM siRNA per well with 10 or 30% FBS. FRET signal of polyplexes incubated with FBS was compared to that of polyplexes in PBS alone. In all cases, black, clear bottom 96 well plates were used for fluorescence measurements. FRET signal was tracked over the course of 100 at 5 min intervals.

Polyplex stability was also evaluated in response to heparin salts. Again, polyplexes were prepared to have final concentrations of 100 nM siRNA per well. In each well, 90 μ L of polyplexes were incubated with 10 μ L of various concentrations of heparin salts, ranging from 20 U/mL to 100 U/mL final concentration heparin per well. FRET signal was then evaluated the same as the above FBS-based method.

Hemolysis Assay

Red blood cell (RBC) hemolysis assay was performed using methods described previously.³⁴ Blood was drawn from consenting human donors according to an IRB-approved protocol. In short, RBCs were isolated from whole blood and diluted into buffer solutions of pH 5.6, 6.2, 6.8, and 7.4. Polyplexes prepared at 5 and 40 μ g/mL polymer concentration were incubated with RBCs at the various pH values for 1 h in round-bottom 96-well plates. Negative controls and positive controls of RBCs in buffer only or Triton-X, respectively, were also used for analysis. The RBCs were then centrifuged, and supernatants were analyzed for absorbance at 450 nm using a plate reader. Percent hemolysis was evaluated by subtracting background buffer-only RBC absorbance from the absorbance of polymer-containing wells and dividing by the difference between Triton-X controls (complete lysis) and buffer-only RBC absorbance (no lysis).

Creation of Luciferase-Expressing MDA-MB-231 Breast Cancer Cells and Luciferase-Expressing NIH3T3 Mouse Fibroblast Cells

Lentivirus was produced by transfecting HEK-293T cells with pGreenFire1-CMV plasmid, along with pMDLg/pRRE, pRSV-Rev, and pMD2.G packaging plasmids with Lipofectamine 2000 as a transfection reagent. Media supernatant containing lentivirus was then collected at 48 and 72 h. For transfection of MDA-MB-231 and NIH3T3 cell lines, 10 mL lentiviral media was added to the cells containing 6 μ g/mL Polybrene for 24 h of incubation. Cells were analyzed post-transduction by detection of GFP using flow cytometry (BD LSR II Flow Cytometer, San Jose, CA, USA). Cells were selected for vector expression by growth in puromycin-containing media.

Cell Culture

All cells used for this manuscript were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco Cell Culture, Carlsbad, CA), containing 4.5 g/L glucose, 10% FBS (Gibco), and 0.1% gentamicin (Gibco).

Cell Viability

Luciferase-expressing NIH3T3 cells or MDA-MB-231 cells were seeded in a 96-well plate at 20,000 cells/mL (2000 cells per well). After 24 h, polyplex solutions were introduced to the wells using an N⁺:P⁻ ratio of 20 with 100 nM scrambled siRNA per well. After 24 h,

polyplex-containing media was removed from the cells and replaced with media containing luciferin substrate (150 $\mu\text{g/mL}$). After incubating for 5 min, cells were imaged using an IVIS Lumina III imaging system (Caliper Life Sciences, Hopkinton, Massachusetts). Luciferin-containing media was then replaced with normal media for 24 h, followed by IVIS imaging with luciferin media at 48 h post-treatment. Luminescence signal was compared to untreated controls for analysis.

***In Vitro* Luciferase Silencing of MDA-MB-231 Cells**

Luciferase-expressing MDA-MB-231 cells were seeded in 96-well plates at 2000 cells per well and allowed to adhere for 24 h. Polyplex solutions containing either luciferase siRNA or scrambled siRNA at 100, 75, 50, 25, 10, and 1 nM were then incubated with MDA-MB-231 cells in quadruplicate. After 24 h, media was replaced with luciferin-containing media (150 $\mu\text{g/mL}$), and luminescence was evaluated by IVIS imaging. Luciferin-containing media was then replaced with normal media until 24 or 48 h, at which point luciferin media was reintroduced, and luminescence again evaluated. For analysis of knockdown, all data were normalized to scrambled control polyplexes to account for any nonspecific toxicity effects. IC_{50} values were calculated using nonlinear regression in Graphpad.

Uptake by MDA-MB-231 Cells

Nonluciferase expressing MDA-MB-231 cells were seeded in 12-well plates at 80,000 cells per well. Polyplexes were formed containing 100 nM of Alexa Fluor-488-labeled nucleic acid in media containing 10% serum. Polyplexes were incubated with cells for 30 min, 4, 24, or 48 h and then removed. Cells were washed with PBS, trypsinized for 10 min in 0.25% trypsin, and centrifuged at 450g for 7 min. Cell pellets were then resuspended in PBS containing 0.04% trypan blue (to quench extracellular fluorescence) prior to running through a flow cytometer (3-laser BD LSR II, BD Biosciences, Franklin Lakes, NJ, USA). Cells were monitored for Alexa Fluor-488 fluorescence at excitation and emission wavelengths of 488 and 519 nm, respectively. Quantification of % uptake was performed using FlowJo software (FlowJo, LLC, Ashland, OR). Events were gated for single cells and then characterized for negative, low, medium, and high uptake levels. Untreated MDA-MB-231 cells were used as negative controls.

Isothermal Titration Calorimetry

Isothermal titration calorimetry experiments were performed using a MicroCal VP-ITC (Malvern, USA) in the Vanderbilt Center for Structural Biology. Polyplexes were prepared at concentrations of 0.5 mg/mL polymer as described above. BSA was dissolved from lyophilized powder at 15 mg/mL in buffer solutions exactly matching the composition of polyplex buffer. Titration experiments were carried out at 37 °C using a reference power of 10 $\mu\text{cal/s}$, 300 s initial delay, and 307 rpm stirring speed. Each injection was 10 μL , with a duration of 20 s, spacing of 260 s, and filter period of 2 s. A control consisting of heat of dilution of BSA into buffer only was subtracted from titration data. All data analysis was performed in Origin, using a one set of sites binding model to determine thermodynamic parameters. A cationic control polymer consisting of PDMAEMA only in the corona, as previously described, was used as a positive control for protein adsorption.^{14,64–66}

Gel Shift Protein Adsorption Assay

Polyplexes bearing scrambled siRNA were prepared at 0.5 mg/mL polymer and incubated with 5% FBS. A 2% agarose gel was prepared using 1× TAE buffer. Ten μL of these preparations each was added to wells on an agarose gel with 2 μL loading dye. Agarose gels were run at 90 mV for 30 min. Gels were then stained using SYPRO Ruby stain according to established commercial protocols (Thermo Fisher) and imaged using a BioRad Chemidoc Imaging System.

Complement Assay

All materials for the hemolysis-based complement assay were purchased from Complement Technologies (Tyler, TX, USA). siRNA polyplexes were prepared at 50 nM siRNA. Complement sera was prepared at five dilutions (1:20, 1:40, 1:80, 1:160, 1:320), and antibody-sensitized sheep RBCs were prepared separately at 2×10^8 cells/mL in GVB⁺⁺ buffer. In each test tube, 100 μL of complement sera was added to 100 μL of polyplexes and incubated for 30 min. Then 100 μL of antibody-sensitized RBCs were added to each tube, and the mixtures were incubated at 37 °C for 1 h with intermittent shaking. All samples were then centrifuged, and supernatants were transferred to a 96-well plate. Absorbance at 541 nm was then measured on a plate reader. Absorbance values were used to determine transmittance and absorption (1-transmittance). Percent lysis was calculated as

$$\frac{(\text{sample absorption} - \text{absorption PBS only})}{(\text{absorption water control}[\text{complete lysis}] - \text{absorption PBS only})} \times 100$$

Percent lysis at each complement dilution was plotted and compared to control samples containing complement proteins only (no polyplexes).

Polyplex Pharmacokinetics, Biodistribution, and Intravital Microscopy

In Vivo Polyplex Preparation—For *in vivo* polyplex preparations, polymers were complexed with 1 mg/kg Cy5-labeled oligonucleotides in 100 mM pH4 citrate buffer. Complexing solutions were then loaded into 20 kDa MWCO dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA) and dialyzed into PBS—/— overnight. Polyplex formation was confirmed by dynamic light scattering (described above) immediately prior to *in vivo* injections.

Intravital Microscopy—Male CD-1 mice (Charles River) ($n = 4$ per group) were anesthetized using isoflurane and immobilized on a heated confocal microscope stage. Prior to imaging, mouse ears were cleaned with a depilatory cream. Microscope immersion fluid was used to immobilize the mouse ear on a glass coverslip. Intravital microscopy was performed using a Nikon Czi+ system with a Nikon Eclipse TioE inverted microscopy base, Plan ApoVC 20× differential interference contrast N2 objective, 0.75 NA, Galvano scanner, and 543 dichroic mirror. All image analysis and acquisition were done using Nikon NIS-Elements AR version 4.30.01. A laser gain of 98 was used throughout. Ear veins were detected using the light microscope, and images were focused to the plane of greatest vessel width, where flowing RBCs were clearly visible. Once the ear was in focus, microscope was

switched to confocal laser mode and set to image continuously every second. The mouse was then injected with 100 μL polyplex solution via tail vein at a 1 mg/kg dose (1.89 $\mu\text{mol/kg}$ polymer, corresponding to 54.0, 81.8, 64.4, 85.0, 64.5, and 80.4 mg/kg for 5k PEG, 20k PEG, 10k PMPC, 20k PMPC, 10k POEGMA, and 20k POEGMA, respectively), and Cy5 fluorescence in ear veins was monitored for 20 min. For image analysis, initial background fluorescence was subtracted, and circular regions of interest were highlighted within the mouse ear vessels. Fluorescence from these regions of interest was quantified, and background fluorescence was subtracted. Intensity values were normalized to initial peak intensity. Fluorescence decay curves were modeled as one-compartment systems using single-phase exponential decay. Area under the curve values were calculated by multiplying normalized intensity curves by the dose of siRNA in mg and integrating over time. Pharmacokinetic parameters were calculated using Graphpad Prism analysis software.

Biodistribution—After 20 min of monitoring *via* intravital microscopy, animals were sacrificed. Organs were removed and immediately imaged for Cy5 fluorescence using an IVIS system. Fluorescence was quantified using an IVIS Lumina Imaging system (Xenogen Corporation, Alameda, CA, USA) at excitation and emission wavelengths of 620 and 670 nm, respectively, using Living Image software version 4.4.

***In Vivo* Tumor Gene Silencing**

Athymic female nude mice (4–6 weeks old, Jackson Laboratory) were injected under anesthesia in the mammary fatpad on each side with 1×10^6 luciferase expressing MDA-MB-231 cells in a 50:50 mixture of Matrigel:DMEM (serum-free). Tumor growth was followed until they reached approximately 100 mm³. Polyplexes were prepared and loaded with either luciferase or scrambled siRNA at 1 mg/kg as described for pharmacokinetic studies. Animals were injected i.p. with luciferin substrate (150 mg/kg), imaged for baseline tumor bioluminescence using an IVIS system, and then subsequently injected with polyplexes *via* tail vein. Mice were re-injected with luciferin substrate on days 1, 3, 5, 7, and 10 post-polyplex treatment. The luminescence signal of each individual tumor was compared to its baseline pretreatment signal, and each relative luminescence value was normalized to the average relative luminescence of respective scrambled siRNA polyplex groups ($n = 6$ –10 tumors per group). Body weight measurements of all mice were recorded every day of the study period to monitor toxicity. Of 30 mice studied, five tumor-bearing mice were removed from the study due to health concerns prior to the end of the study, but there were no statistically significant differences in survival between treatment groups.

Biodistribution of Tumor-Bearing Mice

Biodistribution studies for athymic female nude tumor-bearing mice (Jackson Laboratory) were conducted using the same methods as biodistribution studies for the male CD-1 mice. Fluorescence was measured in heart, lungs, kidneys, liver, spleen, and tumors excised at 24 h post-tail vein injection.

***In Vivo* Polyplex Uptake by MDA-MB-231 Breast Tumors**

Tumors isolated from mice during above-described biodistribution experiments were then used for flow cytometry studies of polyplex uptake. Tumors were cut into small pieces,

washed with HBSS containing Ca^{2+} and Mg^{2+} , and then processed using an enzyme mix containing collagenase (0.5 mg/mL, Roche Life Sciences, Indianapolis, IN, USA) and DNase (0.19 mg/mL, BioRAD, Hercules, CA, USA) in DMEM. After 1 h incubation in the enzyme mix, the tumors were centrifuged and resuspended in HBSS without Ca^{2+} and Mg^{2+} and then incubated with 5 mM EDTA for 20 min. Tumors were then centrifuged, and the pellets were resuspended in HBSS with Ca^{2+} and Mg^{2+} and filtered using a 70 μm Nylon cell strainer. Filtrate was then washed once more with HBSS containing Ca^{2+} and Mg^{2+} and then incubated in ACK lysis buffer (Thermo Fisher Scientific, USA) for 2 min before being diluted in 20 mL of PBS $^{-/-}$. Cells were then pelleted and resuspended in 1–2 mL PBS $^{-/-}$ prior to running on a flow cytometer (BD LSRii, BD Biosciences, San Jose, CA, USA). Uptake analysis was performed in FlowJo. Cell populations were isolated using forward and side scatter, then GFP positive tumor cells were gated, and Cy5 fluorescence intensity was measured for the GFP positive tumor cell population.

Tumor Penetration Assays

Ex Vivo Tumor Penetration—Athymic nude mice bearing MDA-MB-231 orthotopic mammary tumors were sacrificed when tumors reached 200–300 mm³. Tumors were then incubated for 24 h in DMEM containing 10% FBS, 1% gentamicin, and polyplexes bearing 100 nM Cy5-siRNA. After 24 h, tumors were embedded in OCT compound (Fisher Healthcare) and frozen on dry ice. Tissues were fully faced and then cryo-sectioned 100 μm deeper in the tissue. Frozen slides were then stained with DAPI and imaged using confocal microscopy (same instrument from intravital microscopy methods). Tissues were analyzed for Cy5 fluorescence by normalizing untreated tumors to account for background autofluorescence. Line profiles were drawn blinded to Cy5 fluorescence in regions where a clearly defined tumor edge was visible. Quantitative analysis consisted of nine line profiles sampled randomly from tumor perimeter, with three tumors per polyplex.

In Vivo Tumor Penetration—Athymic nude mice bearing MDA-MB-231 orthotopic mammary tumors were injected with 1 mg/kg Cy5-siRNA polyplex solutions (at same polymer concentrations as for tumor uptake and intravital microscopy studies). Animals were sacrificed 24 h after injections, and tumors were processed by freezing in OCT as described in the Ex Vivo Penetration Assays section. Tumor sections were imaged using confocal microscopy, and images were sampled randomly throughout interior of tumor section, collecting both Cy5 and DAPI signal.

Statistical Methods

Unless otherwise noted, all statistical determinations were made using one-way ANOVA with Tukey multiple comparisons test in Graphpad Prism. All reported data display mean and standard error. Significance was determined using $\alpha = 0.05$.

Ethics Statement

All animal studies were conducted according to the National Institutes of Health guidelines for care and use of laboratory animals. Each study involving animals in this work was reviewed and approved by the Institutional Animal and Use Committee (IACUC) at Vanderbilt University.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Vanderbilt School of Engineering, funding from National Institutes of Health R01 EB019409-01 and DOD CDMRP OR 130302, and by a National Science Foundation Graduate Research Fellowship under grant no. 1445197. Dynamic light scattering measurements and TEM were performed using instrumentation in the Vanderbilt Institute of Nanoscale Sciences and Engineering. Isothermal titration calorimetry measurements were performed using the Vanderbilt Center for Structural Biology core facilities. We acknowledge the Vanderbilt Translational Pathology Shared Resource supported by NCI/NIH Cancer Center Support grant 2P30 CA068485-14 for their assistance preparing frozen tumor histology sections. The authors acknowledge D. Stec for NMR guidance. Authors additionally acknowledge S. Dudzinski, P. Patil, and I. Kelly for their assistance in tumor processing.

References

1. Ozcan G, Ozpolat B, Coleman RL, Sood AK, Lopez-Berestein G. Preclinical and Clinical Development of siRNA-Based Therapeutics. *Adv Drug Delivery Rev.* 2015; 87:108–119.
2. Zuckerman JE, Davis ME. Clinical Experiences with Systemically Administered siRNA-Based Therapeutics in Cancer. *Nat Rev Drug Discovery.* 2015; 14:843–856. [PubMed: 26567702]
3. Ozpolat B, Sood AK, Lopez-Berestein G. Nanomedicine Based Approaches for the Delivery of siRNA in Cancer. *J Intern Med.* 2010; 267:44–53. [PubMed: 20059643]
4. Nichols JW, Bae YH. Odyssey of a Cancer Nanoparticle: From Injection Site to Site of Action. *Nano Today.* 2012; 7:606–618. [PubMed: 23243460]
5. Alexis F, Pridgen E, Molnar LK, Farokhzad OC. Factors Affecting the Clearance and Biodistribution of Polymeric Nano-particles. *Mol Pharmaceutics.* 2008; 5:505–515.
6. Naeye B, Deschout H, Caveliers V, Descamps B, Braeckmans K, Vanhove C, Demeester J, Lahoutte T, De Smedt SC, Raemdonck K. *In Vivo* Disassembly of IV Administered siRNA Matrix Nanoparticles at the Renal Filtration Barrier. *Biomaterials.* 2013; 34:2350–2358. [PubMed: 23261216]
7. Zuckerman JE, Choi CH, Han H, Davis ME. Polycation-siRNA Nanoparticles Can Disassemble at the Kidney Glomerular Basement Membrane. *Proc Natl Acad Sci U S A.* 2012; 109:3137–3142. [PubMed: 22315430]
8. Pelaz B, del Pino P, Maffre P, Hartmann R, Gallego M, Rivera-Fernández S, de la Fuente JM, Nienhaus GU, Parak WJ. Surface Functionalization of Nanoparticles with Polyethylene Glycol: Effects on Protein Adsorption and Cellular Uptake. *ACS Nano.* 2015; 9:6996–7008. [PubMed: 26079146]
9. Owens DE Iii, Peppas NA. Opsonization, Biodistribution, and Pharmacokinetics of Polymeric Nanoparticles. *Int J Pharm.* 2006; 307:93–102. [PubMed: 16303268]
10. Walkey CD, Olsen JB, Guo H, Emili A, Chan WCW. Nanoparticle Size and Surface Chemistry Determine Serum Protein Adsorption and Macrophage Uptake. *J Am Chem Soc.* 2012; 134:2139–2147. [PubMed: 22191645]
11. Aggarwal P, Hall JB, McLeland CB, Dobrovolskaia MA, McNeil SE. Nanoparticle Interaction with Plasma Proteins as it Relates to Particle Biodistribution, Biocompatibility and Therapeutic Efficacy. *Adv Drug Delivery Rev.* 2009; 61:428–437.
12. Gref R, Lück M, Quéllec P, Marchand M, Dellacherie E, Harnisch S, Blunk T, Muller RH. ‘Stealth’ Corona-Core Nanoparticles Surface Modified by Polyethylene Glycol (PEG): Influences of the Corona (PEG chain length and surface density) and of the Core Composition on Phagocytic Uptake and Plasma Protein Adsorption. *Colloids Surf, B.* 2000; 18:301–313.
13. Perry JL, Reuter KG, Kai MP, Herlihy KP, Jones SW, Luft JC, Napier M, Bear JE, DeSimone JM. PEGylated PRINT Nanoparticles: The Impact of PEG Density on Protein Binding, Macrophage Association, Biodistribution, and Pharmacokinetics. *Nano Lett.* 2012; 12:5304–5310. [PubMed: 22920324]

14. 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]
15. Zhang Y, Xiao C, Ding J, Li M, Chen X, Tang Z, Zhuang X. A Comparative Study of Linear, Y-shaped and Linear-Dendritic Methoxy Poly(Ethylene Glycol)-Block-Polyamidoamine-Block-Poly-(L-Glutamic Acid) Block Copolymers for Doxorubicin Delivery *In Vitro* and *In Vivo*. *Acta Biomater*. 2016; 40:243–253. [PubMed: 27063495]
16. Tockary TA, Osada K, Chen Q, Machitani K, Dirisala A, Uchida S, Nomoto T, Toh K, Matsumoto Y, Itaka K, Nitta K, Nagayama K, Kataoka K. Tethered PEG Crowdedness Determining Shape and Blood Circulation Profile of Polyplex Micelle Gene Carriers. *Macromolecules*. 2013; 46:6585–6592.
17. Moghimi SM, Szebeni J. Stealth Liposomes and Long Circulating Nanoparticles: Critical Issues in Pharmacokinetics, Opsonization and Protein-Binding Properties. *Prog Lipid Res*. 2003; 42:463–478. [PubMed: 14559067]
18. Mishra S, Webster P, Davis ME. PEGylation Significantly Affects Cellular Uptake and Intracellular Trafficking of Non-Viral Gene Delivery Particles. *Eur J Cell Biol*. 2004; 83:97–111. [PubMed: 15202568]
19. Schlenoff JB. Zwitteration: Coating Surfaces with Zwitterionic Functionality to Reduce Nonspecific Adsorption. *Langmuir*. 2014; 30:9625–9636. [PubMed: 24754399]
20. Sin MC, Chen SH, Chang Y. Hemocompatibility of Zwitterionic Interfaces and Membranes. *Polym J*. 2014; 46:436–443.
21. Jin Q, Chen Y, Wang Y, Ji J. Zwitterionic Drug Nanocarriers: A Biomimetic Strategy for Drug Delivery. *Colloids Surf, B*. 2014; 124:80–86.
22. Zou H, Wang Z, Feng M. Nanocarriers with Tunable Surface Properties to Unblock Bottlenecks in Systemic Drug and Gene Delivery. *J Controlled Release*. 2015; 214:121–133.
23. Lewis AL, Tolhurst LA, Stratford PW. Analysis of a Phosphorylcholine-Based Polymer Coating on a Coronary Stent Pre-and Post-Implantation. *Biomaterials*. 2002; 23:1697–1706. [PubMed: 11922473]
24. Bakhai A, Booth J, Delahunty N, Nugara F, Clayton T, McNeill J, Davies SW, Cumberland DC, Stables RH. The SV Stent Study: A Prospective, Multicentre, Angiographic Evaluation of the BiodivYsio Phosphorylcholine Coated Small Vessel Stent in Small Coronary Vessels. *Int J Cardiol*. 2005; 102:95–102. [PubMed: 15939104]
25. Ukawa M, Akita H, Masuda T, Hayashi Y, Konno T, Ishihara K, Harashima H. 2-Methacryloyloxyethyl Phosphorylcholine Polymer (MPC)-Coating Improves the Transfection Activity of GALA-Modified Lipid Nanoparticles by Assisting the Cellular Uptake and Intracellular Dissociation of Plasmid DNA in Primary Hepatocytes. *Biomaterials*. 2010; 31:6355–6362. [PubMed: 20537380]
26. Yu H, Zou Y, Jiang L, Yin Q, He X, Chen L, Zhang Z, Gu W, Li Y. Induction of Apoptosis in Non-Small Cell Lung Cancer by Downregulation of MDM2 using pH-Responsive PMPC-b-PDPA/siRNA Complex Nanoparticles. *Biomaterials*. 2013; 34:2738–2747. [PubMed: 23352573]
27. 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; 7:8870–8880. [PubMed: 24041122]
28. Uddin MJ, Werfel TA, Crews BC, Gupta MK, Kavanaugh TE, Kingsley PJ, Boyd K, Marnett LJ, Duvall CL. Fluorocoxib A Loaded Nanoparticles Enable Targeted Visualization of Cyclooxygenase-2 in Inflammation and Cancer. *Biomaterials*. 2016; 92:71–80. [PubMed: 27043768]
29. Roy D, Berguig GY, Ghosn B, Lane D, Braswell S, Stayton PS, Convertine AJ. Synthesis and Characterization of Transferrin-Targeted Chemotherapeutic Delivery Systems Prepared *via* RAFT Copolymerization of High Molecular Weight PEG Macromonomers. *Polym Chem*. 2014; 5:1791–1799. [PubMed: 25221630]

30. Crownover E, Duvall CL, Convertine A, Hoffman AS, Stayton PS. RAFT-Synthesized Graft Copolymers that Enhance pH-Dependent Membrane Destabilization and Protein Circulation Times. *J Controlled Release*. 2011; 155:167–174.
31. Swierczewska M, Lee KC, Lee S. What is the Future of PEGylated Therapies? *Expert Opin Emerging Drugs*. 2015; 20:531–536.
32. Colombo C, Gatti S, Ferrari R, Casalini T, Cuccato D, Morosi L, Zucchetti M, Moscatelli D. Self-Assembling Amphiphilic PEGylated Block Copolymers Obtained through RAFT Polymerization for Drug-Delivery Applications. *J Appl Polym Sci*. 2016; 133:43804.
33. Liu M, Leroux JC, Gauthier MA. Conformation–Function Relationships for the Comb-Shaped Polymer POEGMA. *Prog Polym Sci*. 2015; 48:111–121.
34. Evans BC, Nelson CE, Yu SS, Beavers KR, Kim AJ, Li H, Nelson HM, Giorgio TD, Duvall CL. Ex Vivo Red Blood Cell Hemolysis Assay for the Evaluation of pH-Responsive Endo-somolytic Agents for Cytosolic Delivery of Biomacromolecular Drugs. *J Visualized Exp*. 2013; 73:50166.
35. Albanese A, Tang PS, Chan WC. The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems. *Annu Rev Biomed Eng*. 2012; 14:1–16. [PubMed: 22524388]
36. Huang R, Lau BL. Biomolecule-Nanoparticle Interactions: Elucidation of the Thermodynamics by Isothermal Titration Calorimetry. *Biochim Biophys Acta, Gen Subj*. 2016; 1860:945–956.
37. Sanchez-Moreno P, Buzon P, Boulaiz H, Peula-Garcia JM, Ortega-Vinuesa JL, Luque I, Salvati A, Marchal JA. Balancing the Effect of Corona on Therapeutic Efficacy and Macrophage Uptake of Lipid Nanocapsules. *Biomaterials*. 2015; 61:266–278. [PubMed: 26005765]
38. Schöttler S, Becker G, Winzen S, Steinbach T, Mohr K, Landfester K, Mailänder V, Wurm FR. Protein Adsorption is Required for Stealth Effect of Poly(Ethylene Glycol)- and Poly-(Phosphoester)-Coated Nanocarriers. *Nat Nanotechnol*. 2016; 11:372–377. [PubMed: 26878141]
39. Walkey CD, Chan WC. Understanding and Controlling the Interaction of Nanomaterials with Proteins in a Physiological Environment. *Chem Soc Rev*. 2012; 41:2780–2799. [PubMed: 22086677]
40. Loizou E, Triftaridou AI, Georgiou TK, Vamvakaki M, Patrickios CS. Cationic Double-Hydrophilic Model Networks: Synthesis, Characterization, Modeling and Protein Adsorption Studies. *Biomacromolecules*. 2003; 4:1150–1160. [PubMed: 12959578]
41. Bartlett DW, Davis ME. Physicochemical and Biological Characterization of Targeted, Nucleic Acid-Containing Nanoparticles. *Bioconjugate Chem*. 2007; 18:456–468.
42. Nomoto T, Matsumoto Y, Miyata K, Oba M, Fukushima S, Nishiyama N, Yamasoba T, Kataoka K. *In Situ* Quantitative Monitoring of Polyplexes and Polyplex Micelles in the Blood Circulation Using Intravital Real-Time Confocal Laser Scanning Microscopy. *J Controlled Release*. 2011; 151:104–109.
43. Jones SW, Roberts RA, Robbins GR, Perry JL, Kai MP, Chen K, Bo T, Napier ME, Ting JPY, DeSimone JM, Bear JE. Nanoparticle Clearance is Governed by Th1/Th2 Immunity and Strain Background. *J Clin Invest*. 2013; 123:3061–3073. [PubMed: 23778144]
44. Kim HJ, Ishii T, Zheng M, Watanabe S, Toh K, Matsumoto Y, Nishiyama N, Miyata K, Kataoka K. Multifunctional Polyion Complex Micelle Featuring Enhanced Stability, Targetability, and Endosome Escapability for Systemic siRNA Delivery to Subcutaneous Model of Lung Cancer. *Drug Delivery Transl Res*. 2014; 4:50–60.
45. Oe Y, Christie RJ, Naito M, Low SA, Fukushima S, Toh K, Miura Y, Matsumoto Y, Nishiyama N, Miyata K, Kataoka K. Actively-Targeted Polyion Complex Micelles Stabilized by Cholesterol and Disulfide Cross-Linking for Systemic Delivery of siRNA to Solid Tumors. *Biomaterials*. 2014; 35:7887–7895. [PubMed: 24930854]
46. Christie RJ, Matsumoto Y, Miyata K, Nomoto T, Fukushima S, Osada K, Halnaut J, Pittella F, Kim HJ, Nishiyama N, Kataoka K. Targeted Polymeric Micelles for siRNA Treatment of Experimental Cancer by Intravenous Injection. *ACS Nano*. 2012; 6:5174–5189. [PubMed: 22575090]
47. Bartlett DW, Su H, Hildebrandt IJ, Weber WA, Davis ME. Impact of Tumor-Specific Targeting on the Biodistribution and Efficacy of siRNA Nanoparticles Measured by Multimodality *In Vivo* Imaging. *Proc Natl Acad Sci U S A*. 2007; 104:15549–15554. [PubMed: 17875985]
48. Dahlman JE, Barnes C, Khan OF, Thiriot A, Jhunjhunwala S, Shaw TE, Xing Y, Sager HB, Sahay G, Speciner L, Bader A, Bogorad RL, Yin H, Racie T, Dong Y, Jiang S, Seedorf D, Dave A, Singh

- Sandhu K, Webber MJ, et al. In Vivo Endothelial siRNA Delivery Using Polymeric Nanoparticles with Low Molecular Weight. *Nat Nanotechnol.* 2014; 9:648–655. [PubMed: 24813696]
49. Huang Y, Lin D, Jiang Q, Zhang W, Guo S, Xiao P, Zheng S, Wang X, Chen H, Zhang HY, Deng L, Xing J, Du Q, Dong A, Liang Z. Binary and Ternary Complexes Based on Polycaprolactone-Graft-Poly (N, N-Dimethylaminoethyl Methacrylate) for Targeted siRNA Delivery. *Biomaterials.* 2012; 33:4653–4664. [PubMed: 22480869]
 50. Zuckerman JE, Gritli I, Tolcher A, Heidel JD, Lim D, Morgan R, Chmielowski B, Ribas A, Davis ME, Yen Y. Correlating Animal and Human Phase Ia/Ib Clinical Data with CALAA-01, a Targeted, Polymer-Based Nanoparticle Containing siRNA. *Proc Natl Acad Sci U S A.* 2014; 111:11449–11454. [PubMed: 25049380]
 51. Sarett SM, Werfel TA, Chandra I, Jackson MA, Kavanaugh TE, Hattaway ME, Giorgio TD, Duvall CL. Hydrophobic Interactions between Polymeric Carrier and Palmitic Acid-Conjugated siRNA Improve PEGylated Polyplex Stability and Enhance *In Vivo* Pharmacokinetics and Tumor Gene Silencing. *Biomaterials.* 2016; 97:122–132. [PubMed: 27163624]
 52. Liu X, Chen Y, Li H, Huang N, Jin Q, Ren K, Ji J. Enhanced Retention and Cellular Uptake of Nanoparticles in Tumors by Controlling Their Aggregation Behavior. *ACS Nano.* 2013; 7:6244–6257. [PubMed: 23799860]
 53. Liu X, Li H, Chen Y, Jin Q, Ren K, Ji J. Mixed-Charge Nanoparticles for Long Circulation, Low Reticuloendothelial System Clearance, and High Tumor Accumulation. *Adv Healthcare Mater.* 2014; 3:1439–1447.
 54. Wang J, Yuan S, Zhang Y, Wu W, Hu Y, Jiang X. The Effects of Poly(Zwitterions)s versus Poly(Ethylene Glycol) Surface Coatings on the Biodistribution of Protein Nanoparticles. *Biomater Sci.* 2016; 4:1351–60. [PubMed: 27426309]
 55. Zhou W, Shao J, Jin Q, Wei Q, Tang J, Ji J. Zwitterionic Phosphorylcholine as a Better Ligand for Gold Nanorods Cell Uptake and Selective Photothermal Ablation of Cancer Cells. *Chem Commun.* 2010; 46:1479–1481.
 56. Tu S, Chen YW, Qiu YB, Zhu K, Luo XL. Enhancement of Cellular Uptake and Antitumor Efficiencies of Micelles with Phosphorylcholine. *Macromol Biosci.* 2011; 11:1416–1425. [PubMed: 21793214]
 57. Chen L, Wang H, Zhang Y, Wang Y, Hu Q, Ji J. Bioinspired Phosphorylcholine-Modified Polyplexes as an Effective Strategy for Selective Uptake and Transfection of Cancer Cells. *Colloids Surf, B.* 2013; 111:297–305.
 58. Murdoch C, Reeves KJ, Hearnden V, Colley H, Massignani M, Canton I, Madsen J, Blanz A, Armes SP, Lewis AL, MacNeil S, Brown NJ, Thornhill MH, Battaglia G. Internalization and Biodistribution of Polymersomes into Oral Squamous Cell Carcinoma Cells *In Vitro* and *In Vivo*. *Nanomedicine.* 2010; 5:1025–1036. [PubMed: 20874018]
 59. Eliyahu G, Kreizman T, Degani H. Phosphocholine as a Biomarker of Breast Cancer: Molecular and Biochemical Studies. *Int J Cancer.* 2007; 120:1721–1730. [PubMed: 17236204]
 60. Chen Q, Osada K, Ge Z, Uchida S, Tockary TA, Dirisala A, Matsui A, Toh K, Takeda KM, Liu X, Nomoto T, Ishii T, Oba M, Matsumoto Y, Kataoka K. Polyplex Micelle Installing Intracellular Self-Processing Functionalities without Free Cationomers for Safe and Efficient Systemic Gene Therapy through Tumor Vasculature Targeting. *Biomaterials.* 2017; 113:253–265. [PubMed: 27835820]
 61. Hansen AE, Petersen AL, Henriksen JR, Boerresen B, Rasmussen P, Elema DR, Rosenschöld PMA, Kristensen AT, Kjær A, Andresen TL. Positron Emission Tomography Based Elucidation of the Enhanced Permeability and Retention Effect in Dogs with Cancer Using Copper-64 Liposomes. *ACS Nano.* 2015; 9:6985–6995. [PubMed: 26022907]
 62. Miller MA, Gadde S, Pfirschke C, Engblom C, Sprachman MM, Kohler RH, Yang KS, Laughney AM, Wojtkiewicz G, Kamaly N, Bhonagiri S, Pittet MJ, Farokhzad OC, Weissleder R. Predicting Therapeutic Nanomedicine Efficacy using a Companion Magnetic Resonance Imaging Nanoparticle. *Sci Transl Med.* 2015; 7:314ra183.
 63. Clark AJ, Wiley DT, Zuckerman JE, Webster P, Chao J, Lin J, Yen Y, Davis ME. CRLX101 Nanoparticles Localize in Human Tumors and Not in Adjacent, Nonneoplastic Tissue after Intravenous Dosing. *Proc Natl Acad Sci U S A.* 2016; 113:3850–3854. [PubMed: 27001839]

64. Convertine AJ, Benoit DS, Duvall CL, Hoffman AS, Stayton PS. Development of a Novel Endosomolytic Diblock Copolymer for siRNA Delivery. *J Controlled Release*. 2009; 133:221–229.
65. Nelson CE, Gupta MK, Adolph EJ, Shannon JM, Guelcher SA, Duvall CL. Sustained Local Delivery of siRNA from an Injectable Scaffold. *Biomaterials*. 2012; 33:1154–1161. [PubMed: 22061489]
66. Nelson CE, Kim AJ, Adolph EJ, Gupta MK, Yu F, Hocking KM, Davidson JM, Guelcher SA, Duvall CL. Tunable Delivery of siRNA from a Biodegradable Scaffold to Promote Angiogenesis *In Vivo*. *Adv Mater*. 2014; 26:607–614. [PubMed: 24338842]

Figure 1.

siRNA polyplexes containing varied corona architectures. All polymers contain the same polyplex core-forming block consisting of equimolar DMAEMA and BMA. The corona-forming blocks comprise either linear PEG, zwitterionic PMPC, or brush PEG structures (POEGMA), as pictured. Polymer structures are displayed on the left, with the core-forming block in red and corona-forming block in blue. Polymers are complexed with siRNA at low pH, triggering spontaneous assembly of polyplexes before the pH is raised to physiological pH.

Figure 2.

Polyplexes with different corona chemistries have similar size, zeta potential, and cargo loading but varied stability against high salt concentrations. (A, B) Polyplex siRNA encapsulation efficiency and stability is highest at $N^+:P^-$ 20. (A) Ribogreen assay reveals polyplex encapsulation plateaus by $N^+:P^-$ ratio of 10. (B) Polyplexes retain higher stability after a 30 min incubation in 30% FBS at $N^+:P^-$ 20 compared to $N^+:P^-$ 10 ($p < 0.01$, $n = 3$). (C) All polyplexes were around 100–145 nm in average size. (D–I) Dynamic light scattering traces show that 20k PMPC and 20k PEG populations are more resistant to high salt conditions ($N^+:P^-$ 20).

Figure 3.

The 20k PEG and 20k PMPC corona-forming blocks increase polyplex stability in heparin salts relative to all other formulations. Polyplexes were also incubated for 100 min in (A) 20, (B) 60, and (C) 100 U/mL heparin salts. 20k PMPC and 20k PEG maintained the greatest stability levels at each heparin condition. All measurements represent average of three separate stability experiments. All polyplexes were formulated at $N^+:P^-$ 20.

Figure 4.

In vitro, all tested polyplex surface chemistries, except POEGMA, exhibited desirable hemolysis, cytocompatibility, uptake, and target gene knockdown properties. All assays were performed at a dose of 100 nM siRNA and 2.7 μ M polymer ($N^+ : P^-$ 20) unless otherwise noted. (A) In a RBC hemolysis assay, all polyplexes (at 40 μ g/mL polymer) displayed similar pH-dependent membrane disruptive behavior well-tuned for endosomal escape due to their consistent core-forming polymer block composition which dictates this behavior. (B) Polyplex viability in NIH 3T3 fibroblasts at 100 nM siRNA 48 h after polyplex addition. (C) All high molecular weight coronas had significantly lower uptake compared to lower molecular weight counterparts, ($p < 0.01$, $n = 3$). (D) Percent of cells in low, medium, and high gates for each polyplex. 20k PEG polyplexes had the highest percent of cells in the low uptake group compared to any other polyplex ($p < 0.001$, $n = 3$). (E) Dose–response curve of polyplex inhibition of luciferase activity. Luciferase activity was monitored after 48 h polyplex treatment. (F) IC₅₀ values for all polyplexes reveal significantly improved efficacy of PMPC-based polyplexes compared to PEGylated polyplexes of similar molecular weight ($p < 0.04$, $n = 3$).

Figure 5.

Higher molecular weight coronas reduce albumin adsorption, while none of the polyplexes significantly adsorb complement proteins. (A) Schematic of ITC setup. BSA is slowly titrated into solution of polyplexes, and changes in heat are recorded and used to calculate thermodynamic parameters. (B) ITC of polyplexes (0.5 mg/mL polymer, $N^+ : P^-$ 20) indicated significantly less favorable BSA interaction for 20k PEG and 20k PMPC compared to standard 5k PEG ($n = 3$, $p < 0.05$). (C) Schematic of complement assay setup. (D) Negligible complement protein adsorption was observed for all polyplex surface chemistries, measured by % lysis compared to complement protein controls at various dilutions. Cationic control polyplexes (100% PDMAEMA-based particle surface) served as a positive control for protein/complement adsorption in these assays.

Figure 6.

High molecular weight zwitterionic and linear PEG coronas significantly improve polyplex pharmacokinetics (1 mg/kg Cy5-siRNA, 1.89 μ mol/kg polymer, N⁺:P⁻ 20). (A) A panel of intravital microscopy images for visualization of pharmacokinetic differences between polyplexes shows an obvious increase in circulation time for 20k PEG and 20k PMPC compared to gold standard 5k PEG. (B) Average intravascular fluorescence intensity curves from mouse ear vessel imaging quantify this outcome ($n = 4$). (C) Area under the curve shows that 20k PEG and 20k PMPC had roughly 3- to 4-fold higher bioavailability compared to other polyplexes ($n = 4$, $p < 0.001$).

Figure 7.

Zwitterionic 20k PMPC polyplexes significantly increased luciferase knockdown and siRNA delivery per tumor cell compared to PEGylated polyplexes *in vivo* (1 mg/kg siRNA, 1.89 μ mol/kg polymer, $N^+ : P^-$ 20). (A) 20k PMPC polyplexes decreased tumor luminescence by roughly 80% compared to scrambled control polyplexes over the course of a 10 day study period, with significantly improved knockdown compared to PEGylated polyplexes on days 3–7 (1 mg/kg intravenous siRNA dose on day 0, $n = 6$ –10 tumors per group, $p < 0.05$). (B) Representative IVIS luminescence images taken on day 3 post-treatment. (C) Representative histograms of polyplex uptake for tumor cells analyzed by flow cytometry from tumors harvested at 24 h post-treatment. Results indicate increased Cy5 fluorescence in GFP positive MDA-MB-231 cells for 20k PMPC polyplex-treated tumors compared to PEGylated polyplexes. (D) At 24 h, 20k PMPC polyplexes had significant, 63% increased mean Cy5 fluorescence in tumor cells compared to 20k PEG and 213% relative to 5k PEG ($p = 0.0005$ vs 5k PEG, $p = 0.0526$ vs 20k PEG, $n = 4$ –6 tumors per group). (E) 20k PMPC had significantly increased % positive cells at 24 h compared to 5k PEG ($n = 4$ –6 tumors per group, $p = 0.0114$), while 20k PEG had a strong trend toward increased % positive cells compared to 5k PEG ($p = 0.0582$). (F) In an *in vitro* time course, 20k PMPC polyplexes exhibited significantly higher uptake compared to PEGylated polyplexes at 30 min, 4 h, and

48 h ($p < 0.02$, $n = 3$). (G) Histological quantification of polyplex intensity after 24 h *ex vivo* incubation with MDA-MB-231 tumors excised from athymic nude mice. Line profiles were drawn starting at the tumor edge ($n = 27$, 3 tumors per polyplex, 9 profiles per tumor). (H) Representative histology images of *ex vivo* and *in vivo* tumor penetration of polyplexes. Overall, polyplexes achieved similar penetration depths both *in vivo* and *ex vivo*, but histology evidence supports differences in uptake between polyplex types.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Polymer Molecular Weights (¹H NMR), Monomer Compositions (¹H NMR), and Polydispersity indices (Gel Permeation Chromatography)

polymer	total MW (g/mol)	corona MW (g/mol)	% BMA	% DMAEMA	PDI
5k PEG	28636	5000	50	50	1.021
20k PEG	43353	20000	50	50	1.071
10k PMPC	34129	10177	51	49	<i>a</i>
20k PMPC	45044	21092	50	50	<i>a</i>
10k PEGMA	34171	11716	47	53	1.156
20k PEGMA	42619	18667	49	51	1.296

^a PMPC polymers were not analyzed with GPC due to insolubility in mobile DMF phase.

Table 2
Pharmacokinetic Parameters Quantified from Intravascular Imaging Data Including Half-Life ($T_{1/2}$), Area under the Curve (AUC), and Clearance (Cl)

polymer	$T_{1/2}$ (min)	AUC (mg·min)/(L)	Cl (mL/min)
5k PEG	5.2 ± 1.7	127 ± 37	0.22 ± 0.07
20k PEG	22.4 ± 5.8	507 ± 152	0.06 ± 0.02
10k PMPC	8.4 ± 1.0	192 ± 23	0.13 ± 0.02
20k PMPC	26.3 ± 5.5	586 ± 157	0.05 ± 0.01
10k POEGMA	7.6 ± 0.4	165 ± 14	0.15 ± 0.01
20k POEGMA	7.4 ± 2.0	176 ± 45	0.15 ± 0.04
Cy5 nucleic acid	1.87 ± 0.20	75 ± 1.4	4.20 ± 0.27