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The Effect of N/P Ratio on the *In Vitro* and *In Vivo* Interaction Properties of PEGylated Poly(2-(dimethylamino)ethyl methacrylate)-Based siRNA Complexes

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Abstract

In this study, we employed poly(ethylene glycol)-poly(*n*-butyl acrylate)-poly(2-(dimethylamino)ethyl methacrylate) (PEG-PnBA-PDMAEMA) triblock copolymer micelles as well as a PEG-PDMAEMA diblock copolymer as model systems for studying the role of N/P ratio on the *in vivo* behaviors of PEGylated siRNA carriers in mice. Through various *in vitro* assays, we identified the presence of a free/uncomplexed polymer population coexisting with siRNA complexes, the extent of which was found to be an increasing function of the N/P ratio. Contrary to what one might expect, however, we found that for both the diblock and triblock-based siRNA carrier systems, a change in the N/P ratio exerts insignificant influence on the *in vivo* biodistribution and *ex vivo* blood chemistry properties of the respective systems at < ~ 6 hours after systemic injection in mice. On the other hand, histological analysis of major organs at a longer time point (≈ 16 hours) indicates that the presence of uncomplexed polymer elicits toxicity to the organ that is associated with the clearance of the siRNA complexes from the circulation system. This effect can be eliminated by working at N/P ratios near the charge-neutralization point of the complexes.

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Keywords

biodistribution; blood toxicity; N/P ratio; polyplex; siRNA

1. Introduction

N/P ratio, or basically the ratio of positively-chargeable polymer amine (N = nitrogen) groups to negatively-charged nucleic acid phosphate (P) groups, is perhaps one of the most important physicochemical properties of polymer-based gene delivery vehicles. The N/P character of a polymer/nucleic acid complex can influence many other properties such as its net surface charge, size, and stability. At high N/P ratios, especially ones well above the point required to form charge-neutralized complexes with siRNA, important questions arise about how these complexes will behave in an *in vivo* environment in response to the excess cationic charge. One important implication of N/P ratio in DNA polyplex systems is the enhancement in *in vitro* gene expression^[1–3] that is typically observed at high N/P ratios as a result of free cationic polymer which enhances intracellular delivery^[4–5]. However, the trade-off to higher gene expression is the higher toxicity that accompanies it. It has been identified that the major source of systemic toxicity elicited by DNA polyplexes at high N/P ratios is also the existence of a high excess of free cationic polymer which induces nonspecific aggregation with blood components and leads to capillary occlusions^[6]. When the excess free polymer is purified from the complexes, *in vivo* toxicity is drastically reduced^[6]. To our knowledge, the only published study to date which has attempted to directly address the role of N/P ratio in the delivery performance and toxicity of siRNA/polymer complexes is by Aigner and colleagues^[7]. Using both pristine and PEGylated PEI systems, this group identified key mechanisms involved in polyplex uptake by kidney, lung, liver and spleen (i.e., the four major organs where the polyplex nanoparticles mainly accumulate); while the kidney uptake is largely non-tissue-specific, the uptake by the other organs involves respective tissue-specific biological processes, i.e., aggregation of the polyplexes with erythrocytes for the lung uptake, and phagocytosis of the polyplexes for the uptake by liver and spleen^[7]. Furthermore, this and their other^[8] work also demonstrates that the PEGylated nanoparticles are more clinically-viable delivery vehicles than the basic polycations in terms of blood stability, which is consistent with the previous findings that PEGylation protects and keeps nanoparticles stable^[9–10] and shields positive surface charges to lessen toxicity-inducing nonspecific electrostatic interactions *in vivo*^[11]. In connection with the topic of the present paper, it needs to be pointed out, however, that Aigner and coworkers focus their N/P effect study on the non-PEGylated siRNA delivery system, and they do not report any results on the pharmacokinetics and *in vivo* biodistribution behavior of the potentially more important PEGylated PEI-siRNA complexes as a function of N/P ratio. Also, to our knowledge, there has been no previous study that touches on how different complex architectures (e.g., micelles, which are becoming an increasingly popular platform for nucleic acid delivery^[12–15]) will perform *in vivo* at various N/P ratios compared to conventional polyplexes. In the present work, we have employed as model PEGylated systems, a diblock copolymer, PEG-PDMAEMA, and micelles prepared from the triblock copolymer PEG-PnBA-PDMAEMA. PDMAEMA-based polymers have been commonly reported throughout the literature as successful intracellular delivery vehicles for siRNA and

DNA^[16–18] Recent experiments indicate that PDMAEMA has a higher proton buffering capacity than PEI^[19–21]. The current study explores for the first time the role of N/P ratio on the *in vivo* behavior of PEGylated PDMAEMA-based polyplexes and micelle-siRNA complexes. It will be shown that a “free polymer” population which is believed to be linked to the possible systemic toxicity associated with high N/P ratio nanoparticles manifests itself differently for these PEGylated PDMAEMA systems than what has been observed previously with the non-PEGylated PEI system^[7].

2. Experimental Section

2.1 Materials

Coomassie Brilliant Blue R-250 (Cat # 161-0400) was purchased from BioRad Laboratories. 10% washed, pooled, bovine erythrocytes were purchased from Rockland Immunochemicals (Gilbertsville, PA) and stored at 4 °C until use. The siRNAs materials used were purchased from Bioneer, Inc. (Daejeon, Korea). The blood chemistry reagents were purchased from Fujifilm (Japan).

The polymers used in this study, PEG₁₁₃-PDMAEMA₁₄₂ (the subscript numbers denote the number-average degrees of polymerization of the individual blocks, M_n (overall number-average molecular weight for the copolymer) = 26,220 g/mol, PDI (overall polydispersity index of the copolymer) = 1.23) and PEG₁₁₃-PnBA₁₀₀-PDMAEMA₁₂₆ (M_n = 32,600 g/mol, PDI = 1.29) were synthesized by atom transfer radical polymerization (ATRP) starting from a PEG macroinitiator (M_n = 5,000 g/mol, PDI = 1.07, purchased from Polysciences, Inc.) with a brominated end functionality and the final products were characterized for molecular weight and polydispersity by proton nuclear magnetic resonance (¹H NMR) spectroscopy and gel permeation chromatography (GPC), respectively. Full synthesis and characterization details for these polymers have been reported previously^[22]

2.2 Methods

Coomassie Blue/Ethidium Bromide Costaining Technique for Quantitative Gel Analysis—1% (w/v) agarose gels were used to examine the migration patterns of pure polymer or micelles and their siRNA complexes at a range of N/P ratios. Ethidium bromide (EtBr) (0.004% v/v) was added to molten agarose before the gel solidified. After the gel was cast, samples to be analyzed were mixed with Blue Juice (Invitrogen) and loaded into separate lanes and electrophoresed in TAE buffer for 1 hour at 3.4 V/cm. Afterwards, the gel was placed on a UV transilluminator to view the EtBr-containing bands, and images were taken with a Polaroid gel camera using 667 film (VWR). Next, gels were rinsed with DI water and immediately transferred into a bath of Coomassie Blue staining solution (0.2% (w/v) in a 50% methanol, 40% water, 10% acetic acid buffer). The gels were stained in Coomassie Blue for at least 6 hours at room temperature, under gentle rocking to ensure uniform staining. Gels were then destained twice to remove the blue background allowing clearer visualization of stained bands. The first destaining step was 10–15 minutes in a 50% methanol, 40% water, 10% acetic acid buffer, under gentle rocking, and the second destaining step was overnight in a 10% methanol, 10% acetic acid, 80% water buffer, under gentle rocking. The bath containing the staining and destaining solutions was also covered

with aluminum foil to suppress evaporation of methanol. Following destaining, the bands stained with Coomassie Blue were visualized on a white light box and imaged with a digital camera.

Quantification of band intensities was performed in ImageJ software (NIH). Color images were first converted to 16-bit black and white images. Next, using the selection tool, a rectangular box of a set size was drawn on the gel starting at an arbitrary reference point before the loading wells, encompassing the entire band of interest, and extending a few units of measure beyond the band. Finally, using the “plot profile” function, the pixel intensity for a given band was plotted as a function of distance along the gel. This procedure (utilizing the same reference points and rectangle size) was repeated until all bands on the gel were quantified.

Based on the intensity profiles (pixel intensity as a function of distance along the gel), band migration distances were calculated using a simple quantitative analysis. First, for each image (e.g., diblock complexes at N/P 2–10 acquired under UV light), a pixel intensity threshold was chosen, below which was considered to be background noise and above which was considered real data. Threshold values were needed in order to determine where the actual baseline of the profiles began and distinguish them from background noise caused by unrelated nonuniformities in the gel itself. Incorporating this threshold across all samples analyzed from the same image, the actual distances corresponding to the terminal ends of the intensity distributions were interpolated from the data.

Dynamic Light Scattering in the Presence of Bovine Serum Albumin—15 μ l samples of PEG-PDMAEMA diblock copolymer, PEG-PnBA-PDMAEMA triblock copolymer micelles and their complexes with siRNA (termed polyplexes and micelleplexes, respectively) at N/P 4 and 8 were prepared in 10 mM Tris-HCl buffer. An aliquot of 50 mg/ml BSA stock solution (purchased from USB, Cleveland, OH) was then added (along with additional 10 mM Tris-HCl, pH 7.5 to make a final BSA concentration of 34.3 mg/ml) to the samples which were subsequently mixed by pipetting up and down ten times and left to sit for 5 minutes before measuring the size. The change in size of the complexes was monitored in a low-volume, quartz cuvette, as a function of time, using DLS on a Malvern Zetasizer Nano S. Hydrodynamic diameters were calculated from the size distribution, unless noted otherwise, by volume (generated by the Non-Negatively Constrained Least Squares (NNLS) method for polydisperse samples), provided by the Malvern software, and are reported as the average of at least 3 independent measurements $\pm$ the standard deviation.

Bovine Erythrocyte Aggregation Study—PEG-PDMAEMA diblock copolymer and PEG-PnBA-PDMAEMA micelle complexes with a negative control siRNA were prepared at N/P ratios 2, 3, 4, 5, 8 and 10 by diluting 5 μ g of siRNA and the appropriate amount of polymer with 10 mM Tris-HCl buffer to a final volume of 100 μ l. The 100 μ l of complexes were then added to 200 μ l of 0.1% (v/v) bovine erythrocytes (diluted with CMF saline from the 10% stock purchased from Rockland Immunochemicals, Gilbertsville, PA) in a 12-well tissue culture plate. The aggregation behavior of the erythrocytes in the presence of the complexes at various N/P ratios was then monitored as a function of time by optical microscopy at 40 $\times$ magnification. The main time points studied were 0, $\frac{1}{2}$, 1, 2 and 3 hours,

however, additional 4, 5 and 6 hour time points were conducted for N/P 3 and 4, because these conditions exhibited interesting, time-dependent behavior where the erythrocytes were stable up to the 3 hour mark, but showed signs of aggregation thereafter.

Animal Care Procedures—Five-week-old male BALB/c-nude mice were purchased from Orient Bio, Inc. (Seongnam, Korea), and used at the age of 8 weeks in the *in vivo* biodistribution study. C57BL/6 mice were originally purchased, also from the same vendor, bred as a homozygous inbred line, and used at 8 weeks of age in the *ex vivo/in vivo* blood chemistry and tissue histology studies. All mice were housed and handled in a specific pathogen-free environment within the animal facility of the Samsung Biomedical Research Institute (SBRI) at the Samsung Medical Center (SMC) (Seoul, Korea), in accordance with the Institute of Laboratory Animal Resources (ILAR) guidelines of the Association for the Accreditation of Laboratory Animal Care (AAALAC) International; the SBRI Animal Facility is an AAALAC International-accredited facility. All animal protocols were reviewed and approved by the Institutional Animal Care & Use Committee (IACUC) of the SBRI.

In Vivo Toxicity Following Systemic Administration of Complexes—*In vivo* toxicity studies were conducted 16 hours after injection of the nanoparticles into tail vein of C57BL/6 mice (8 weeks old; $n = 3$). After blood was collected from the major saphenous vein, mice were sacrificed and major organs were harvested, including lung, liver, spleen and kidney. The organs were fixed with 4% formaldehyde for three days. After dehydration of the fixed tissues, standard histological procedures were used to examine histopathological changes in these organs. 5–6 μm thick paraffin slides were stained with hematoxylin and eosin (H & E) for visual histological evaluation. The tissue slides were digitally scanned using a scanner and, if necessary, further inspected at high resolution.

For blood chemistry tests, serum was separated from whole blood by centrifugation at 1500 rpm for 10 minutes. Each serum sample was then analyzed for the levels of GOT (glutamic oxaloacetic transaminase, in units of mmol of p-nitrophenol produced by 1 l of serum), GPT (glutamic pyruvic transaminase, in units of mmol of p-nitrophenol produced by 1 l of serum), HDL (high-density lipoprotein, mg/dl), TCHO (total cholesterol, mg/dl), and TG (triglycerides, mg/dl) using a FUJIFILM Dri-Chem FDC 3500s biochemical analyzer.

Ex Vivo Blood Toxicity—Hematologic toxicities of the complexes were evaluated using mouse blood samples collected from the major saphenous vein. These blood samples were then stabilized with 1 mM EDTA and stored in glass tubes until free polymer or siRNA/polymer complexes were added to them, and the resultant mixtures were incubated for one hour at room temperature under constant shaking before being analyzed for blood toxicity. Specifically, eight different groups ($n=3$ for each group) were prepared by mixing 0.5 ml of the EDTA-stabilized mouse blood with 0.25 ml of solutions containing the following respective components: (i) 64.13 μg of free PEG-PDMAEMA, (ii) 104.53 μg of free PEG-PnBA-PDMAEMA, (iii) PEG-PDMAEMA-based siRNA complexes at N/P 8 at a total siRNA amount of 1.0 nmol (i.e., 64.13 μg of the diblock copolymer, and 13.00 μg of siRNA), (iv) PEG-PDMAEMA-based siRNA complexes at N/P 4 at a total siRNA amount of 1.0 nmol (i.e., 32.01 μg of the diblock copolymer, and 13.00 μg of siRNA), (v) PEG-

PDMAEMA-based siRNA complexes at N/P 4 at a total siRNA amount of 2.0 nmol (i.e., 46.13 μg of the diblock copolymer, and 26.00 μg of siRNA), (vi) PEG-PnBA-PDMAEMA-based siRNA complexes at N/P 8 at a total siRNA amount of 1.0 nmol (i.e., 104.53 μg of the triblock copolymer, and 13.00 μg of siRNA), (vii) PEG-PnBA-PDMAEMA-based siRNA complexes at N/P 4 at a total siRNA amount of 1.0 nmol (i.e., 52.27 μg of the triblock copolymer, and 13.00 μg of siRNA), and (viii) PEG-PnBA-PDMAEMA-based siRNA complexes at N/P 4 at a total siRNA amount of 2.0 nmol (i.e., 104.53 μg of the triblock copolymer, and 26.00 μg of siRNA). PBS was used as a negative control. After the one-hour incubation, complete blood count (CBC) tests were conducted to evaluate the impact of the free polymer or the polymer/siRNA complexes on the CBC profile, using a hematology analyzer (Cell Dyne 3500R SL, Abbott Laboratories). Acronyms used throughout the text are defined as follows: WBC (white blood cell count, in units of $\times 10^3$ cells per μl of blood), RBC (red blood cell count, $\times 10^6$ cells per μl of blood), HGB (hemoglobin concentration, g/dl), HCT (hematocrit, %), MCV (mean corpuscular volume, femtoliters or fl), MCH (mean corpuscular hemoglobin, picograms or pg), MCHC (mean corpuscular hemoglobin concentration, g/dl), RDW (red cell distribution width, %), PLT (platelet count, $\times 10^3$ cells per μl of blood), MPV (mean platelet volume, fl), PCT (platelet crit, %), PDW (platelet distribution width, %), NEU (number of neutrophils, %), LYM (number of lymphocytes, %), MONO (number of monocytes, %), EOS (number of eosinophils, %), and BASO (number of basophils, %).

Statistical Analysis—*P* values were calculated from the data using a two-sided student's *t*-test. Significance is indicated when $P < 0.05$.

In Vivo Biodistribution—Iodine¹²⁴-labeled siRNA was synthesized as follows. The Bolton-Hunter reagent (i.e., the water non-soluble form of N-succinimidyl-3-(4-hydroxyphenyl)propionate, SHPP) was purchased from Pierce Biotechnology. The radioactive isotope of iodine, I¹²⁴ ($t_{1/2} = 4.2$ days), was provided by the Korea Institute of Radiological and Medical Sciences (KIRAMS). 10.0 μl of SHPP dissolved in DMSO (8 mg/ml) was added to a weakly acidic (pH 6.0) I¹²⁴ solution. 10.0 μl of Chloramine-T (Sigma) solution (10 mg/ml) was added to the above mixture. This final mixture was incubated for 15 seconds to allow the complete oxidation of the iodine. The SHPP-coupled I¹²⁴ product was extracted with a 40:1 (v/v) mixture of benzene and DMF. The organic extract layer was collected into a microcentrifuge tube, and the organic solvent was removed by evaporation under continuous nitrogen flow. Amine-modified siRNA (synthesized from Samchully Pharm., Korea) dissolved at a concentration of 100 nmol/ml in 50 mM aqueous sodium borate (pH 8.5) was added to the above SHPP-I¹²⁴ solution and allowed to react for one hour. The final I¹²⁴-siRNA product was carefully purified by dialysis. The amount of I¹²⁴ attached to siRNA was measured to be around 100–133 μCi (microcuries, a unit of radioactivity) per nmol of siRNA.

Small animal PET-computed tomography (PET-CT) imaging was performed with an Inveon microPET-CT scanner (Siemens) at Samsung Biomedical Research Institute (Korea). Right before imaging, tumor-bearing BALB/c-nude mice (8 weeks old; $n = 3$ for all samples except the naked siRNA control for which $n = 1$; see the last paragraph below for

preparation details) were anesthetized with 1% isoflurane breathing tube on a heated (30 °C) pad. 100–200 μCi of I^{124} -labeled siRNA/polymer (or siRNA/ micelle) complexes was injected via tail vein. Immediately after the micro-PET scan, mice were subjected to a 10-min micro-CT scan, using standard image acquisition parameters. Static micro-PET scans were acquired at 1.5 and 6 hours post-injection with micro-CT scan for anatomical coregistration. To determine temporal changes of tracer concentration in various tissues, ellipsoid or activity-guided, user-defined regions of interest were placed in the region that exhibited organ-characteristic I^{124} activity as determined by visual inspection. To minimize partial volume effects between tissue types, care was taken not to use overlapped borders between organs. Considering the size of the studied organs, tumors and the spatial resolution of the PET scanner, the partial volume effects are, even if they existed, expected to have a very minor impact on the results of quantitative analysis. Activity concentrations are shown as the percentage of the decay-corrected injected activity divided by the mass of the studied organ (% injection dose per gram or %ID/g).

PC9 non-small cell lung adenocarcinoma cell line was kindly provided by Kazuto Nishio (Japan). PC9 cells were cultured and maintained in RPMI 1640 medium (Gibco, USA) containing 1% penicillin-streptomycin and 10% fetal bovine serum. Exponentially growing 10^6 PC9 cells in 100 μl of Matrigel (BD Biosciences, USA) were injected subcutaneously into the lower right back of BALB/c-nude mice. Xenograft animal models were used for in vivo experiments, when tumor volumes reached approximately 50–100 mm^3 (typically 2–3 weeks after the implantation).

3. Results and Discussion

Effect of N/P Ratio on the Charge Properties of the Polyplexes

Due to the presence of the cationic PDMAEMA block in both the PEG-PDMAEMA diblock copolymer and the PEG-PnBA-PDMAEMA triblock micelles, electrostatically-driven complexation can occur spontaneously between polymer and anionic siRNA. If the amount of siRNA is fixed, and the amount of added polymer is systematically increased, this serves to increase the “N/P ratio” or the ratio of nitrogens (from the polymer) to phosphates (from siRNA) in the system. Hence, although 100% of the amine groups of the polymers will not be charged at physiological pH (≈ 7.4 ; note the monomer pK_a of PDMAEMA is known to be 8.4^[19]), increasing the N/P ratio does increase the net amount of cationic charges in the system. Figure S1 shows gel electrophoresis data demonstrating the charge characteristics of the diblock complexes and triblock micelleplexes at various N/P ratios. For the diblock complexes, N/P 2 is approximately the point at which enough polymer has been added to neutralize all of the original siRNA and thus the charge-neutral complexes remain close to the loading position. At all N/P ratios above ~ 2 (i.e., at N/P 5, 8, 10 and 15), the diblock complexes migrate towards the anode. Migration towards the anode at high N/P ratios is suspected to be a result of the net surface charge of the entities in the system becoming more positive as increasing amounts of polymer is added to the system. The micelleplexes become neutralized at an N/P ratio between 2 and 5 (DLS measurements indicate that stable-sized micelleplexes form at N/P 4, data not shown) but the complexes do not migrate outside of the loading position even at high N/P ratios. This is most likely an artifact caused by the

large size of the micelleplexes restricting their movement through the pores of the agarose gel.

Effect of N/P Ratio on the Size Characteristics of the Polyplexes

The hydrodynamic sizes of the pure diblock and triblock polymers as well as their complexes with siRNA at N/P 4 and N/P 8 were measured by DLS; as discussed above (and also as will be discussed later with reference to Figure 3), the N/P 4 condition was chosen to represent a (close-to) charge-neutralized situation where the amount of free unbound polymer is negligible, whereas the N/P 8 condition represents a situation where there is a significant amount of excess polycations that remain uncomplexed in the solution. The results of these measurements are summarized in Table 1, and representative size distributions (by volume) and correlation functions for each sample as determined by DLS are also presented in Figures S2 and S3 of the Supporting Information (SI). As can be seen in Table 1, the triblock micelles were observed to be on the order of 50 nm-diameter particles, and the sizes of the triblock micelle/siRNA complexes at both N/P ratios of 4 and 8 were comparable to that of the micelle precursor within statistical uncertainties. The diblock copolymer chains are about 15 nm in hydrodynamic diameter, and the diblock-based siRNA complexes at both the N/P ratios also showed only small (perhaps statistically insignificant) differences from the size of the pristine diblock copolymer. Cryo-TEM and fluid AFM studies of the micelles and micelleplexes have been performed, and representative images have been reported previously elsewhere^[23].

Effect of N/P Ratio on the Compositions of the Polyplexes

In order to independently study the electrophoretic migration patterns of both pure, uncomplexed polymer as well as siRNA/polymer complexes, a method needed to be developed to separately label polymer molecules and siRNA molecules. Ethidium bromide was an obvious choice for siRNA staining due to its availability and ease of use for staining nucleic acids in agarose gels. Silver staining (i.e., staining by complexation of silver with neutral amines), although shown to be effective for labeling amine-containing molecules^[24–25] is a labor-intensive process involving numerous staining and destaining reagents and developing solutions, and in the end the sensitivity of detection for synthetic polymers is not known. One advantage of the PDMAEMA polymer systems is that they all contain cationic amine groups which have the potential for binding with an oppositely-charged dye molecule. Coomassie Blue is one such anionic dye molecule which is capable of labeling positively-charged molecules (such as polymers) via electrostatic interactions with their cationic amine groups. In the literature, there are a few published reports^[26–27] of successful labeling of amine-based dendrimers with Coomassie Blue in gel electrophoresis and the sensitivity of detection, $\sim 1.5 \mu\text{g}$ ^[27] seemed feasible for this study. The technique employed in the current study was to run samples of polymer and complexes on a gel pre-stained with ethidium bromide, view the siRNA-containing bands on a UV transilluminator, and then finally stain the gel with a Coomassie Blue solution, and view the polymer-containing bands on a white light box. With this costaining technique, it was possible to view, for a given sample, how far the uncomplexed polymer migrated with respect to the siRNA-bound polymer. In the case of polymer which was present only in the form of a complex with siRNA, the band visualized with Coomassie Blue staining should have

migrated exactly as far as the band visualized by ethidium bromide staining. In the case where some of the polymer exists in the form of a complex with siRNA, and some polymer remains free and unbound, we would expect to see the band visualized with Coomassie Blue staining to have migrated further towards the anode (indicating a higher net positive charge) than the band visualized with ethidium bromide (EtBr) staining. As we can see from Figure 1 (top panel), which shows an overlay comparison of gels stained with ethidium bromide and Coomassie Blue, the diblock complexes clearly contain some amount of uncomplexed polymer, the proportion of which increases significantly as the N/P ratio is increased from the charge-neutralization onset (≈ 2) to higher values. A quantitative analysis of the band intensities as a function of distance along the gel, demonstrating an N/P-dependent change in migration length for Coomassie-stained bands compared to EtBr-stained bands is shown in Figure S4 and tabulated in Table 2. It is notable that at N/P ratios greater than or equal to 4, although the migration distances of the EtBr and Coomassie-stained bands both increase as functions of N/P ratio, their ratio remains nearly at the same level (≈ 1.2). This trend is what we would expect if we consider that, above the critical charge-neutralization N/P ratio, the amount of complexed diblock polymers will increase with increasing N/P ratio by the same proportion as the amount of free (uncomplexed) chains; this result is perhaps indicative of the reversible nature of the PDMAEMA-siRNA binding process.

The gel migration behavior of the PEG-PnBA-PDMAEMA micelleplex system characterized using the costaining technique was seen to be different than that of the PEG-PDMAEMA diblock polyplex system (see Figures 1 (bottom) and S5, and Table 2). Because of their large size and bulky architecture (as we have observed previously in siRNA complexation studies) the micelles and micelleplexes do not migrate far outside of the loading well, making it impossible to observe any differences in the migration pattern of the micelleplexes at various N/P ratios. However, based on the chemical similarity of the PDMAEMA blocks involved in the electrostatic complexation process, it follows by extension from the diblock case that the triblock micelleplexes also contain increasing amounts of free PDMAEMA chains at high N/P ratios. It should be clarified, however, that in the micelleplex case, each micelle is estimated to contain, on average, about 173 PDMAEMA brush chains, so it would be difficult to imagine that there exists any micelle totally free of siRNA attached to it even at reasonably high N/P ratios (e.g., at N/P = 8). Now that we have identified the coexistence of both free polymer and siRNA-complexed polymer in high N/P ratio samples, the following sections will explore the consequences of this free polymer population on the behavior of the overall system.

Effect of N/P Ratio on the Interactions of the Polyplexes with Serum Proteins Measured *In Vitro*

In the physiological conditions *in vivo*, synthetic polymer complexes have to encounter many charged species during the journey through the blood stream to their final destination inside of a cell. Negatively-charged blood components, particularly large, macromolecular proteins like serum albumin, can interact non-specifically with cationic polymers to form large aggregates which can significantly impact both the cell-level and systemic-level siRNA delivery properties of the polymers. Particularly at high N/P ratios where there is likely to be a large excess of free cationic chains, these non-specific interactions are

expected to be even more operative than at N/P ratios closer to the neutralization point of the complexes, where the excess charges are minimal. To determine what effect, if any, N/P ratio had on the likely “*in vivo* size” of complexes made with the PEG-PDMAEMA diblock copolymer and the PEG-PnBA-PDMAEMA triblock micelles, we exposed the complexes at N/P 4 and N/P 8 to physiological levels (~ 34 mg/ml)^[28] of bovine serum albumin (BSA, hydrodynamic diameter $\approx 6 \pm 1$ nm (as determined by DLS; data not shown), 69 kilodaltons^[29]) over 90 minutes and monitored the hydrodynamic diameter of the complexes as a function of time. The DLS profiles for the diblock complexes and micelleplexes (in 10 mM Tris-HCl, pH 7.5) for the 90 minute period of exposure to BSA are shown in Figures 2(A) and 2(B), respectively, with the average sizes of the complexes during BSA exposure summarized in Table S1.

As can be seen from Figure 2(B), the first important feature of the size profiles is that the increase in size as a result of BSA interactions is not time-dependent. In other words, aggregates form within the first few seconds/minutes of exposure and do not worsen as time goes on. Secondly, in the case of micelleplexes, the extent of aggregation does not appear to be N/P ratio dependent, as the pure micelles, and micelleplexes at N/P 4 and N/P 8 all consistently form aggregates of 100–150 nm at every time points. Aggregates of this size range, < 150 nm, should not affect the uptake process, as they are still within the limits necessary for endocytosis. The fact that neither pure micelles nor micelleplexes at N/P 8 (which should have more net positive charges in the system) experience more aggregation than micelleplexes at N/P 4 appears to suggest that the complexed siRNA molecules are reasonably uniformly distributed throughout the whole micelle population, and therefore even at the lower N/P ratio of 4:1, each micelle particle still has a sufficient number of uncomplexed PDMAEMA segments that are available to bind BSA, leading to the formation of BSA-mediated, small-size (100 – 150 nm diameter) clusters of the micelleplex particles. We note that in Figure 2(B) (and also in Figure 2(A)) the small-sized particles having 5 – 6 nm diameter represent unbound BSA molecules.

As can be seen in Figure 2(A), the aggregation behavior of the diblock complexes was found to be more telling about the presence of a free polymer population. While the size of N/P 4 complexes (medium-sized, red circles) remains relatively unchanged over the duration of the experiment, suggesting that they experience very little if any aggregation, the N/P 8 complexes (medium-sized, 10 – 30 nm in diameter, purple squares) increase in size by about 100% after BSA exposure. Interestingly, the pure, uncomplexed diblock also increases in size by about 100% after BSA exposure. The behavior of the N/P 8 complexes closely mimicking that of the pure diblock suggests that there is a significant amount of excess, uncomplexed diblock present at N/P 8 and that there is likely to be an N/P dependence to the severity of aggregation. This aggregation effect highlights the potential for non-specific interactions of complexes at high N/P ratios with serum proteins and other negatively-charged molecules *in vivo*. It should be noted that the intensity distribution shows a population of large-sized particles (>100 nm) present in all samples at all time points. However, when considered in volume terms (note $I \sim R^6$ where I and R respectively denote the Rayleigh scattering intensity and the size of the scatterer), these large particles constitute only an insignificant portion (i.e., less than 6% by volume) of the overall population, and

therefore they are not deemed to be very representative of the overall behavior of the complexes.

Effect of N/P Ratio on the Interactions of the Polyplexes with Erythrocytes Measured *In Vitro*

Red blood cells, also known as erythrocytes, have a negative surface charge due to the presence of sialic acid groups in their membranes^[30] and thus are susceptible to non-specific interactions with positively-charged components flowing through the blood stream. As demonstrated by gel electrophoresis in the previous section, adding increasing amounts of polymer to siRNA creates a more and more cationic environment as the N/P ratio is increased. Regardless of whether the added polymer goes on to form part of a complex or if it exists free in solution, the net cationic charge of the solution increases as a function of N/P ratio. Thus, it is expected that the tendency of the complexes to interact/agglomerate with negatively-charged blood cells will also increase with N/P ratio. An eventual result of severe erythrocyte aggregation *in vivo* may be toxicity to the host, in the form of occlusions to capillaries in lungs^[6, 31] and other organs which could result in organ damage or even death. Thus, it is very important to understand the implications of using cationic polymers *in vivo* at various N/P ratios from the context of not only performance, but also short and long-term toxicity.

Figure S6 of the SI shows the state of erythrocytes in pure CMF saline over a period of 20 hours, indicating that no non-specific aggregation occurs in the absence of polymer complexes. In Figure 3, we explore the effect of increasing the N/P ratio of PEG-PDMAEMA and PEG-PnBA-PDMAEMA complexes on the tendency to cause aggregation of negatively-charged erythrocytes; also see Figure S7 through Figure S18.

For PEG-PDMAEMA complexes, very slight aggregation was observed at N/P 4 starting at ½ hour and the extent of aggregation remained constant throughout the rest of the experiment. At N/P ratios 5 and above, severe aggregation was observed as early as ½ hour and the extent of aggregation remained fairly constant at all later time points. However, the observed aggregation at N/P 5 was less severe than at N/P 8 (and N/P 10; see Figure S12). No aggregation was observed at N/P 2 for the diblock complexes, consistent with our gel electrophoresis data indicating that neutrality occurs around N/P 2. For the PEG-PnBA-PDMAEMA micelleplexes, no aggregation was observed until N/P 5, where there was moderate aggregation starting at ½ hour but not worsening with time. This behavior is also consistent with our observation that micelleplex neutrality occurs at about N/P 4, so there is not an excess of free cationic charges present at low N/P ratios to cause aggregation. At N/P 8 (and N/P 10; see Figure S18), severe aggregation, of about the same magnitude as seen with the PEG-PDMAEMA diblock complexes under the same conditions was observed.

In general, nonspecific erythrocyte aggregation *in vivo* can be expected for the PDMAEMA-based systems at N/P ratios high above the charge neutralization point, but it can be alleviated or dramatically reduced by working at sufficiently low N/P ratios where there is much less free polymer present in solution (e.g., N/P 4). We have explored the possibility that the observed aggregation at high N/P ratios is simply a result of having a higher concentration of polymer, but determined that even when we prepared complexes at an N/P

ratio of 4 with the equivalent absolute polymer concentration that would be present at N/P 8 (in other words, double both the siRNA and polymer concentrations, but keep their ratio the same), the aggregation observed was consistent with what we would expect from N/P 4 complexes (see Figures S19 and S20 of the SI). Hence, absolute polymer concentration is not the factor driving aggregation, but it is instead the ratio of polymer to siRNA, or more specifically, the charge ratio (N/P) of the solution. This data also allows us to exclude the possibility that the erythrocyte aggregation may have been caused by the so-called depletion effect of the micelleplex/polyplex particles, because in that situation the degree of aggregation is expected to be an increasing function of overall polymer/micelle concentration^[32–33]. It is therefore most likely that the N/P-dependent erythrocyte aggregation resulted from the presence of excess amounts of free polymer at high N/P ratios. This result suggests that N/P ratio may need to be carefully tuned to avoid aggregation with blood cells following systemic injection.

Effect of N/P Ratio on the Short-Term *In Vivo* Blood Toxicity Measured *Ex Vivo*

We have seen from the previous studies that N/P ratio might exert a fairly influential role in the physicochemical properties of complexes. Although these *in vitro* systems can give us an idea of how polymer complexes may interact with biological components (e.g. BSA, erythrocyte aggregation), they are highly artificial compared to what complexes would actually experience in systemic, *in vivo* circulation. To examine the effect of polymer encounter with various blood components, we incubated polymer/siRNA complexes at N/P ratios of 4 and 8 with freshly prepared blood containing 1 mM EDTA (ethylenediaminetetraacetic acid) as anti-coagulant for one hour *ex vivo*; note that as demonstrated earlier with reference to Figure 3, here the N/P = 4 value was chosen to represent the (close-to-)charge-neutral condition at which there exists a negligible amount of free unbound polymer, and the N/P 8 condition was chosen to represent the charge-reversed state where the excess polycations remain uncomplexed in the solution. Figure 4 shows that, in both the polyplex and micelleplex cases, upon encounter of polymer with murine blood, extensive aggregation of monomeric platelets was induced, reflected by marked drop in the number of platelets in the complete blood count (CBC) profile. It also showed that the triblock micelleplexes more strongly induced platelet aggregation compared to the diblock polyplexes. The cause of platelet aggregation is at present not well-understood, though they seem to be independent of a calcium-mediated process, given the presence of 1 mM EDTA in the current *ex vivo* condition^[34]. Interestingly, the red blood cell (RBC) count was not significantly changed upon prolonged incubation with the polyplexes or micelleplexes *ex vivo*, indicating that a measurable amount of erythrocyte aggregation did not occur. In separate *in vitro* experiments, it was found that the addition of 1 mM EDTA completely suppressed aggregation of both diblock and triblock complexes at N/P 8 with erythrocytes over a 20 hour period (see Figure S33 of the SI), which suggests that the presence of EDTA in the *ex vivo* tests was likely the reason for the difference in the RBC aggregation behavior from the *in vitro* results. Most importantly, the amount of platelet aggregation was found to be not altered significantly according to different N/P ratios or different overall polymer content. One possible explanation for this observation is that in contrast to the previous results obtained from purified erythrocytes, the free polymer preferentially interacts with species other than erythrocytes, perhaps with many other smaller components (e.g., proteins,

etc.) that can kinetically out-compete the bulky erythrocytes in the interaction with polymers, and as a result, the initial difference in N/P ratio becomes effectively unnoticeable in the sense of electrostatic interactions of the nanoparticles with the surroundings; it should be noted that on the *in vitro* level the presence of 1 mM EDTA was experimentally determined to have a negligible effect on the aggregation behavior of complexes with BSA (data not shown). It is also noted that for some CBC parameters such as the white blood cell (WBC), neutrophil (NEU), lymphocyte (LYM), monocyte (MONO), eosinophil (EOS) and basophil (BASO) counts, the wide fluctuations in these data appear to indicate that these components also interact upon encounter with nanoparticles *in vivo*. However, in several repeated measurements (data not presented), the relatively large errors associated with the data were consistently reproduced, and we found no evidence of statistically significant trends in the effect of the N/P ratio on these parameters. All other CBC parameters, such as hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), mean platelet volume (MPV), platelet crit (PCT) and platelet distribution width (PDW), were also seen to be unaffected by the diblock polyplexes or triblock micelleplexes.

Effect of the N/P Ratio on the *In Vivo* Biodistribution of the Polyplexes

To determine the biodistribution of systemically injected diblock polyplexes and triblock micelleplexes and to assess the possible effects of different N/P ratios on the pharmacokinetics of these nanoparticles, we traced the distribution of radio-labeled siRNA/complex in mice; the procedure for the labeling of siRNA with ^{124}I is presented in the Methods section. The biodistribution profiles at 1.5 (Figure 5(A)) and 6 hours (Figure 5(B)) post-injection showed that although there existed several minor differences, *in vivo* biodistribution is largely unaffected by N/P ratio; the biodistribution of micelleplexes formulated at N/P 8 is largely comparable to that of N/P 4 complexes. Diblock siRNA complexes at N/P 4 and N/P 8 also displayed similar trends in their biodistribution. The static PET-CT together with early time point dynamic PET data of diblock siRNA nanoparticles (Figure S34 of the SI) suggest very rapid clearance of these nanoparticles *in vivo*, the major route of excretion is through the liver, and that the nanoparticles have a very short half life. In both the polyplex and micelleplex cases, *in vivo* biodistribution was found to be unaffected by the change in the N/P ratio from 4 to 8 units, although the same amount of change in the N/P ratio was observed to cause a dramatic change in the aggregation state of erythrocyte cells (i.e., from well dispersed to aggregated) *in vitro* as discussed in a previous subsection. On the other hand, these biodistribution results appear to be consistent with the results of the *ex vivo* CBC tests (discussed in the immediately previous subsection). It should also be particularly noted that the N/P-independent uptake of the PDMAEMA polyplexes/micelleplexes by the lung tissue is in contrast to what has been previously observed for PEI-based polyplexes; in the PEI systems, the accumulation in the lung *in vivo* shows a strong correlation with the degree of erythrocyte aggregation due to the polyplexes *in vitro*^[7]. Further investigation is required for clarification of the mechanism responsible for this difference.

To further examine the possible effect of N/P ratio on the longer-time *in vivo* behavior of nanoparticle complexes, several different formulations of diblock copolymer polyplexes and triblock copolymer micelleplexes were systemically administered to mice via tail vein injection, and the CBC profiles and the histopathological properties of several major organs were examined at 16 hours after the injection. In contrast to the *ex vivo* CBC results at one hour post-exposure, both the diblock polyplexes and triblock micelleplexes were found to cause no significant change in the CBC profiles of the exposed mice at the 16 hour time point (Figures S21 and S22 of the SI), which, we believe, suggests that the mouse's homeostasis mechanism has completely rebalanced the normal levels of the hematological components by this later time point. In order to determine whether the nanoparticles had any impact on the excretory organs that they fenestrate through during the clearance process, in particular the liver, we analyzed the *in vivo* glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), high-density lipoprotein (HDL), total cholesterol (TCHO) and triglycerides (TG) levels in the bloods of the animals treated with the various types of polymer/siRNA complexes at 16 hours post-injection; these parameters are measures of the possible damage in the hepatic functions caused during the excretion of the nanoparticles. The results shown in Figure 6 indicate no significant alterations in these parameters, and thus no systemic liver toxicity from the functional standpoint in any of the polymer/siRNA-treated mice groups.

Further insight could be obtained by histological tissue analysis. We conducted histopathological examinations of mice's livers and spleens (liver and spleen are two major organs of the reticuloendothelial system (RES)) as well as lungs and kidneys of the tested mice. Both the diblock polyplex and triblock micelleplex-treated groups showed normal lung and kidney tissue structures, and no histopathological changes were detected in these organs relative to those of the control(PBS)-treated mice (Figures S23 through S31 of the SI). Interestingly, while the group of mice treated with the diblock complexes exhibited no considerable change in the liver histology, the liver cells of the micelleplex-treated mice displayed markedly shrunken cytoplasmic contents with significant void volumes. The extent of such effect was highest for the N/P 8 micelleplexes at a total siRNA dose of 1 nmol (Figure 7(A)), followed by the N/P 4 micelleplexes at a total siRNA dose of 2 nmol (Figure 7(B)), whereas the N/P 4 micelleplexes at a total siRNA dose of 1 nmol caused almost no discernible histopathological effect on the liver tissue (Figure 7(C)). The histological alteration observed in the liver is consistent with the observed metabolic fate of the nanoparticles *in vivo*; i.e., the liver was observed to be the major excretory organs for these nanoparticles. It is interesting to note that this liver morphology change was observed at 16 hours post-injection, despite the fact that the vast majority of the nanoparticle population should have already been cleared from the circulation by this time point; for instance, already at 6 hours post-injection only less than a few % of the nanoparticles was estimated to remain circulating in the blood, and by 24 hours virtually no nanoparticle was detected in the circulation (data not shown). Nonetheless, the highest level of liver histological alteration observed at the N/P 8 condition suggests that uncomplexed PDMAEMA segments, while present in the excretory pathway, play an important role in causing histological changes in the liver, although the molecular-level mechanism of this effect of the uncomplexed PDMAEMA segments for liver needs to be further investigated.

We would also like to point out that the histological sections of the spleens of the micelleplex-treated mice appeared to exhibit higher concentrations of dark spherical domains than those taken from the PBS or diblock polyplex-treated mice (Figures S24, S27 and S30 of the SI), which is consistent with previous reports that nanoparticles of sizes in the range 30 – 70 nm are observed to be trapped in the spleen^[35].

4. Conclusions

The evidence from the various experiments performed in this study convincingly indicate that PDMAEMA-based polymers form complexes with siRNA at a critical charge neutralization ratio, and above this ratio, extra polymer added to increase the N/P ratio contributes to the free/uncomplexed polymer population, in addition to further contributing to the structure of the complexes. This conclusion was supported by DLS, in which we observed an increase in the complex size of PEG-PDMAEMA complexes at N/P 8 after exposure to BSA, which was not observed for complexes at N/P 4. Additionally, quantification of intensity profiles of EtBr/Coomassie Blue costained gels confirmed that increasing amounts of free polymer are present for PEG-PDMAEMA complexes as the N/P ratio increases. This trend was also reflected in the erythrocyte aggregation study, wherein we observed an N/P ratio-dependent increase in nonspecific aggregation. However, contrary to what one would expect from these *in vitro* data, we found that for both the diblock and triblock-based siRNA carrier systems, a change in the N/P ratio (i.e., the amount of free, uncomplexed polymers) causes no influence on the *in vivo* biodistribution and *ex vivo* blood chemistry properties of the respective systems at relatively short times (< ~ 6 hours) after systemic injection in mice. We suspect that under *in vivo* conditions the free polymer chains preferentially interact with molecular components of the blood (such as proteins) that kinetically out-compete the cellular components of the blood in interacting with polymers, and as a result, the initial difference in N/P ratio becomes ineffective in the sense of electrostatic interactions of the polymer/siRNA complexes with the surroundings. On the other hand, the results of the organ histological analysis taken at a longer time point ($\approx$ 16 hours) indicate that a high degree of histological alteration is induced by excess free/uncomplexed cationic polymer in the organs that are associated with the pathway of clearance of the siRNA complexes from the circulation system (i.e., liver and spleen). This systemic histological effect caused by complexes at high N/P ratios due to excess uncomplexed cationic segments was, however, seen to be drastically reduced at lower N/P ratios; i.e., the same effect in the liver detected with the micelleplexes at N/P 8 was not observed when the N/P ratio is reduced to 4 at a fixed total injected siRNA amount of 1 nmol. The interesting findings from this study regarding the as-of-present underdefined role of free/uncomplexed polymer in solutions of PEGylated complexes are very important moving forward, in understanding the care that needs to be taken to formulate polymer nanoparticles at appropriate N/P conditions to optimize delivery but also minimize host toxicity, both of which are critical to the clinical success of the system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Overlaid gel images of (top) PEG-PDMAEMA diblock complexes and (bottom) PEG-PnBA-PDMAEA micelleplexes under UV light (light-colored bands from EtBr staining) and under white light (dark-colored bands from Coomassie Blue staining). The horizontal arrowheads indicate the loading well positions.

(A)

(B)

Figure 2.

Condensed presentation of the hydrodynamic diameter data for the diblock (A) and micelles (B) and their N/P 4 and 8 complexes under exposure to serum levels (~ 35 mg/ml) of BSA. Note that the different sizes of the markers, large, medium and small, are meant to depict the populations which constitute the highest, intermediate and lowest percentages of the intensity distribution, respectively. For instance, the lowest-percentage population,

represented by the smallest markers, contributed less than 10% to the overall scattering intensity. Time zero indicates the diameters of the particles before BSA exposure. Note that in both (A) and (B) the small-sized particles having 5 – 6 nm diameter represent unbound BSA molecules. The actual size distribution data from which these summary plots were excerpted are presented in Figures S35 and S36 of the Supporting Information (SI).

Figure 3. Bright field optical micrographs ($348 \mu\text{m} \times 261 \mu\text{m}$) of bovine erythrocytes incubated with (A) PEG-PDMAEMA diblock complexes and (B) PEG-PnBA-PDMAEMA micelleplexes for 3 hours at the indicated N/P ratios. Additional images for the different N/P ratios taken at various time points are presented in Figures S7 through S18 of the Supporting Information.

Figure 4.

Complete blood count (CBC) analysis (n = 3) following 1 hour *ex vivo* incubation with either PBS (control), PEG-PDMAEMA diblock complexes or PEG-PnBA-PDMAEMA micelleplexes at N/P 8 and N/P 4 containing either 1 nmol or 2 nmol of siRNA incubated with murine blood *ex vivo* for 1 hour. Y-axis is in mixed-units, but indicates the levels of the various blood components being analyzed, some of which have been amplified by some factor (e.g., 10, 100 or 1000) so as to be compared on the same scale. Abbreviations for the blood components are defined in the “*Ex Vivo* Blood Toxicity” section of the Methods (Section 2.2). The specific units used for the various parameters are also given in that section.

Figure 5. Full *in vivo* biodistribution profiles at 1.5 (upper) and 6.0 (lower) hours following systemic injection; a combined profile including free siRNA and accumulation in muscle is also presented in Figure S32 of the Supporting Information. The data are mean values from triplicate assays. The error bars represent the standard deviations.

Figure 6.

Key hepatic blood parameters (i.e., the in vivo glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), high-density lipoprotein (HDL), total cholesterol (TCHO) and triglycerides (TG) levels) measured for the mice treated with the various types of polymer/siRNA complexes at 16 hours post-injection.

(A)

(B)

Figure 7.

Histological sections of mouse livers taken at 16 hours after systemic injection of PBS (control) (left), diblock polyplexes (middle) and micelleplexes (right): (A) both complexes at N/P 8 with a total injected siRNA amount of 1 nmol; (B) both complexes at N/P 4 with a total injected siRNA amount of 2 nmol; (C) both complexes at N/P 4 with a total injected siRNA amount of 1 nmol.

Table 1

Summary of the hydrodynamic diameters (based on the volume-weighted size distributions by DLS) of PEG-PDMAEMA and PEG-PnBA-PDMAEMA polymers and complexes. Values are reported as the mean diameter $\pm$ standard deviation from at least 3 independent measurements.

Polymer Type	Pure Polymer	N/P 4 Complexes	N/P 8 Complexes
PEG-PDMAEMA	14 $\pm$ 1 nm	17 $\pm$ 5 nm	10 $\pm$ 1 nm
PEG-PnBA-PDMAEMA	48 $\pm$ 6 nm	45 $\pm$ 1 nm	56 $\pm$ 3 nm

Table 2

Distances migrated by EtBr and Coomassie-stained bands at various N/P ratios (deduced from analysis of the images in Figure 1). The distance ratio is the ratio of the distance traveled by Coomassie-stained bands relative to its EtBr-stained counterpart at the same N/P ratio. The band migration distances were measured from an arbitrarily chosen reference point. So this analysis only serves a qualitative purpose. Also see Figures S4 and S5 for the actual EtBr and Coomassie band intensity distributions as functions of the distance along the gel from which the above data were determined.

Polymer Type	N/P Ratio	EtBr Distance (cm)	Coomassie Distance (cm)	Distance Ratio
PEG-PDMAEMA	2	1.16	1.31	1.12
	4	1.25	1.47	1.17
	8	1.33	1.62	1.21
	10	1.41	1.71	1.21
PEG-PnBA-PDMAEMA	2	2.37	2.23	0.94
	4	2.41	2.21	0.93
	8	2.56	2.28	0.94
	10	2.56	2.40	0.94