Polymeric Nanocarriers for siRNA Delivery to Murine Macrophages

Diane C. Forbes, Nicholas A. Peppas*

This work investigates the interactions of a polycationic nanocarrier with siRNA and with cells in order to better understand the capabilities and limitations of the carrier. The polycationic nanocarriers are cross-linked copolymer nanoparticles synthesized in a single-step reaction using ARGET ATRP (activators regenerated by electron transfer atom transfer radical polymerization). The polycationic nanocarriers efficiently bind siRNA for polymer/siRNA mass ratios less than 1. A method to prepare fluorescently labeled polycationic nanocarriers is presented. The fluorescently labeled polycationic nanocarriers are used to investigate cellular internalization with RAW264.7 murine macrophage cells. Flow cytometry demonstrates that the uptake increased with nanoparticle concentration and incubation time. Confocal microscopy confirmed internalization of fluorescently labeled nanoparticles. The investigation of siRNA-induced knockdown demonstrates that higher concentrations of nanoparticles and siRNA are associated with increased knockdown. For the conditions tested in the knockdown experiments, the ARGET ATRP polycationic nanocarriers outperformed a commercially available Lipofectamine control.

1. Introduction

Biomaterials researchers operate at the interface between biology and materials science in order to create new materials that can aid patients by detecting, treating, or preventing disease. Drug delivery remains an active focus for biomaterials researchers as well as researchers in the area of nanomedicine. Cationic polymers have been investigated as biomaterials for drug delivery of nucleic acids because they can form polyelectrolyte complexes with negatively charged (anionic) nucleic acids, and the complexation can protect the nucleic acid from degradation and may enhance cellular uptake and endosomal escape.[1] Polymers containing DEAEMA (2-(diethylamino)ethyl methacrylate) or the related DMAEMA (2-(dimethylamino)ethyl methacrylate) have been investigated by many groups for the delivery of DNA[2–12] as well as siRNA[6,13–24] to cells (see Tables 1 and 2).

Delivery of siRNA remains a major challenge to researchers seeking to translate the promise of siRNA to alter gene expression from the lab to the clinic. Effective siRNA delivery depends on complex interactions among the siRNA, carrier, and cells of interest. Undesirable cellular toxicity is a common challenge for polycationic carriers, especially since there seems to be a trade-off between transfection efficiency and cellular toxicity.[25] Another challenge for researchers working to design improved delivery vehicles is that the observed transfection efficiency often does not correlate directly with carrier or siRNA uptake;[26] in other words, the carrier with the best transfection efficiency is not necessarily the one with the most efficient cellular uptake. Like many cationic polymers, PDEAEMA and PDMAEMA contain protonatable amine groups that become positively charged in acidic pH values. This pH-responsiveness from high-to-low pH values is characterized by a “pH-dependent hydrophobe-to-hydrophilic phase shift”[27] when going...
from high pH values to low pH values. At low pH values, the amino group of the DEAEMA and DMAEMA is protonated and positively charged. In the case of a cross-linked hydrogel, the repulsion of the positively charged groups results in swelling. The reported values for the pK$_a$ of PDEAEMA range from 7.0–7.3 [28–30] to 7.68 [31] and the reported pK$_a$ of PDMAEMA is 7.5 [2,32].

This work investigates the interactions of a polycationic nanoparticle carrier synthesized by ARGET ATRP (activators regenerated by electron transfer atom transfer radical polymerization) with siRNA cargo and RAW264.7 cell delivery sites. The polycationic nanoparticle and siRNA interactions are probed using a fluorescent assay as well as light scattering techniques, and the polycationic nanoparticle and RAW264.7 cell interactions are investigated using fluorescently labeled nanoparticles combined with confocal microscopy or flow cytometry. Finally, the use of these ARGET ATRP polycationic nanoparticles as siRNA delivery carriers to RAW264.7 cells is investigated through concentration-dependent transfection experiments.

Table 1. Delivery of siRNA using DEAEMA-containing cationic polymers.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Cell type</th>
<th>siRNA</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAEMA-in-water redox free radical emulsion polymerization with EGDMA cross-linking agent</td>
<td>HuH-7</td>
<td>100 nM GL3</td>
<td>[13,33]</td>
</tr>
<tr>
<td>DEAEMA with PEGDMA cross-linking agent with redox free radical emulsion polymerization in water</td>
<td>BSC-40</td>
<td>100 nM</td>
<td>cyclophilin B</td>
</tr>
<tr>
<td>MSN surface-modified with DEAEMA or DMAEMA polymers, synthesized by free radical polymerization in DMF solvent</td>
<td>B16F10</td>
<td>30 nM GAPDH or luciferase</td>
<td>[19]</td>
</tr>
</tbody>
</table>

B16F10, mouse melanoma; BSC-40, primate kidney epithelial; DMF, dimethylformamide; Dual-Glo, luciferase reporter assay (Promega); EGDMA, ethylene glycol dimethacrylate; FLuc, firefly luciferase; HuH-7, human liver cancer; MSN, mesoporous silica nanoparticles; PEG, poly(ethylene glycol); PEGDMA, PEG dimethacrylate.

Table 2. Delivery of siRNA using DMAEMA-containing polymers.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Cell type</th>
<th>siRNA</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMAEMA-b-PEG palm-tree-like complexes (solvent-free ATRP) with non-covalent post-PEGylation</td>
<td>PC3</td>
<td>300 nM VEGF</td>
<td>[14,35]</td>
</tr>
<tr>
<td>P(DMAEMA-b-(DMAEMA-co-PAACoBMA)) (RAFT in DMF)</td>
<td>HeLa</td>
<td>10–100 nM GAPDH</td>
<td>[16,18]</td>
</tr>
<tr>
<td>Micelles of PDMAEMA-b-PCL-b-PDMAEMA (RAFT in toluene)</td>
<td>MDA-MB/PC3</td>
<td>40 nM GFP40/40 nM VEGF</td>
<td>[17]</td>
</tr>
<tr>
<td>MSN surface-modified with DEAEMA or DMAEMA (free radical polymerization in DMF)</td>
<td>B16F10</td>
<td>30 nM GAPDH or luciferase</td>
<td>[19]</td>
</tr>
<tr>
<td>Micelles of PEG-b-PnBA-b-PDMAEMA (ATRP in DMF)</td>
<td>HeLa</td>
<td>30 nM GAPDH</td>
<td>[21]</td>
</tr>
<tr>
<td>q-DMAEMA and PEGMA with PEGDMA cross-linking agent (water-in-oil inverse emulsion AGET ATRP)</td>
<td>S2</td>
<td>9 pmol RLuc (Dual-Glo)</td>
<td>[22]</td>
</tr>
<tr>
<td>DMAEMA and PEGMA monomers with EGDMA cross-linking agent (ATRP in methanol/toluene)</td>
<td>S2</td>
<td>90 μM RLuc</td>
<td>[24]</td>
</tr>
<tr>
<td>Micelles of PEG-b-P(DMAEMA-co-BMA) (RAFT in dioxane)</td>
<td>MDA-MB</td>
<td>100 nM luciferase</td>
<td>[23]</td>
</tr>
</tbody>
</table>

B16F10, mouse melanoma; Dual-Glo, luciferase reporter assay (Promega); DMF, dimethylformamide; GFP, green fluorescent protein; HeLa, human cervical cancer; MDA-MB, human breast cancer; MSN, mesoporous silica nanoparticles; nBA, n-butyl acrylate; PC3, human prostate cancer; PAA, propylacrylic acid; PEGDMA, PEG dimethacrylate; q-, quaternized with ethyl bromide; RAFT, reversible addition-fragmentation chain-transfer; RLuc, Renilla luciferase; S2, fruit fly Schneider 2; VEGF, vascular endothelial growth factor.
2. Experimental Section

2.1. Chemicals

Reagents poly(ethylene glycol)methyl ether methacrylate (PEGMA) solution (Mₙ 2000 for PEG chain, 50 wt% in water), 2-(diethylamino)ethyl methacrylate (DEAEMA), tert-butyl methacrylate (tBMA), tetraethylene glycol dimethacrylate (TEGDMA), ethyl 2-bromoisobutyrate (EBIB), tris(2-pyridylmethyl)amine (TPMA), and acetic acid (AA) were purchased from Sigma–Aldrich. Ethanol, 1 N hydrochloric acid (HCl), sodium chloride, potassium chloride, monosodium phosphate monohydrate, disodium phosphate heptahydrate, and phosphate buffered saline (PBS) were purchased from Fisher Scientific. Copper(II) bromide was purchased from Acros Organics. Ultrapure water was used for all studies. All chemicals were used as received.

2.2. Nanoparticle Synthesis and Purification

The polycationic nanoparticles were synthesized using a previously reported technique. Monomers (DEAEMA, PEGMA, and tBMA), cross-linking agent (TEGDMA), catalyst (CuBr₂), ligand (TPMA), and initiator (EBIB) were combined at molar ratios of 100:10:45:4:0.5:0.5:4. The reagents were added to a surfactant solution (Brij 30) and mixed with an excess of NBD chloride; a typical reaction was 450 mg of dried nanoparticles with 50 mg NBD chloride in 30 ml ethanol. The mixture was allowed to react overnight to form NBD-labeled nanoparticles (NBD-NPs) (Figure 1). Purification was done by a technique described previously by Fisher and Peppas with repeated precipitation/resuspension with acetone/water) at a 0.1 weight ratio of monomer to solvent. An emulsion was formed using probe sonication (S-4000 Misonix Ultrasonicator, Misonix, Inc.) and then purged with nitrogen. Degassed AA solution was added as a reducing agent (AA:DEAEMA::0:5:100) to start the reaction and the emulsion was allowed to react for 3 h. Purification was done by a technique described previously by Fisher and Peppas with repeated precipitation/resuspension with acetone/0.5 N HCl. Following dialysis (12 000–14 000 molecular weight cut off regenerated cellulose tubing, Spectra/Por), polymer was recovered by freeze-drying.

Fluorescently labeled nanoparticles were synthesized using NBD chloride (NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, also called 4-chloro-7-nitrobenzofuran, Sigma–Aldrich). Nanoparticles containing primary amine were synthesized with 2-aminooctyl methacrylate (AEMA, Sigma–Aldrich) (AEMA:DEAEMA::32·100). Dried, purified nanoparticles were suspended in ethanol and combined with an excess of NBD chloride; a typical reaction was 450 mg of dried nanoparticles with 50 mg NBD chloride in 30 ml ethanol. The mixture was allowed to react overnight to form NBD-labeled nanoparticles (NBD-NPs) (Figure 1). Unreacted NBD-Cl was removed by dialysis and the fluorescently labeled nanoparticles were recovered by freeze-drying. Note that the NBD-Cl becomes strongly fluorescent upon reaction with amines and that NBD-amines are excited by visible light (464 nm) and emit light at 528 nm.

2.3. Binding of siRNA

Nanoparticles were loaded with AllStars Negative Control siRNA (Qiagen) in 1× PBS pH 5.5. Concentrated stock solutions of polycationic nanoparticles in nuclease free water and siRNA in nuclease free water were combined with nuclease free 10× PBS stock solution and nuclease free water to achieve the desired concentrations. During preparation, the solutions were kept on ice. The final concentration of siRNA was maintained at 100 nM as the final concentration of polycationic nanoparticles was decreased by halves from 0.1 mg ml⁻¹ to 6.1 mg ml⁻¹ (g polymer/g siRNA from 80 to 0.00488). The siRNA concentration was maintained constant to minimize variation associated with the assay at low or high concentrations of siRNA. Nuclease free 10× PBS was prepared by dissolving sodium chloride, potassium chloride, monosodium phosphate monohydrate, and disodium phosphate heptahydrate (Fisher Scientific) in water, treating with 0.1% v/v diethylpyrocarbonate (DEPC, Fisher Scientific) overnight, and then autoclaving to remove DEPC. The siRNA/polycationic nanoparticles in 1× PBS pH 5.5 were incubated for 10 min at room temperature prior to evaluating the binding of siRNA.

The fraction of bound siRNA was determined using a Quant-iT RiboGreen RNA Assay Kit (Life Technologies) that was adapted for 384-well low volume plates. Briefly, a 10 µl sample was combined with 10 µl RiboGreen assay solution (RiboGreen reagent diluted 200× in 1× TE buffer) in a black 384-well low volume plate at room temperature. The fluorescence intensity (F) was measured using a microplate reader (Synergy HT, BioTek Instruments, Inc.) 2–5 min after adding the RiboGreen assay solution with 485 nm excitation and 528 nm emission. The RiboGreen reagent is sensitive to components other than RNA present in the assay (although the importance of these contributions diminished for increasing siRNA concentrations), so these contributions to the fluorescence were subtracted from the measured fluorescence signal to calculate the percent loading (L) as calculated in Equation 1:

\[
L = 100 \times \left(1 - \frac{F_{\text{nanoparticle-siRNA complex}} - F_{\text{nanoparticle only}}}{F_{\text{siRNA only}} - F_{\text{buffer only}}} \right)
\]

2.4. Dynamic Light Scattering

The z-average diameter and zeta potential for unlabeled, unfiltered polycationic nanoparticles were measured using a Malvern ZetaSizer NanoZS instrument (Malvern Instruments Corp.) equipped with a 633 nm laser source. Various polycation nanoparticles to siRNA ratios (g polymer/g siRNA) were prepared in nuclease free 0.1× PBS pH 5.5; the polymer nanoparticle concentration was fixed at 0.0125 mg ml⁻¹ and the concentration of siRNA varied (0, 12.5, 50, 200, and 800 nM). The polymer concentration was fixed in order to...
to minimize variation in the light scattering results associated with the concentration of polycationic nanoparticles. Concentrations of siRNA greater than 800 nM were not tested due to the limitations of the stock solution; as a result, the minimum polymer/siRNA mass ratio tested was 1.25.

2.5. Cell Culture

Murine macrophage RAW264.7 cells (obtained from American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium high glucose without L-glutamine (DMEM, Sigma–Aldrich) supplemented with with 1% L-glutamine (MediaTech), 1% penicillin (Sigma–Aldrich), 1% streptomycin (Sigma–Aldrich), and 10% HyClone USDA tested fetal bovine serum (FBS, Thermo Scientific). Opti-MEM reduced serum medium, no phenol red (Life Technologies) was used for all cytotoxicity and transfection experiments.

2.5.1. Flow Cytometry

Flow cytometry measurements were collected using an Accuri flow cytometer and analyzed using Accuri software (BD Biosciences) and also with a BD Fortessa flow cytometer and analyzed using FACSDiva software (BD Biosciences). 240 000 RAW264.7 cells per well were plated in 6-well plates (Thermo Scientific) and incubated for 50 h. Fluorescently labeled polycationic nanoparticles were added at 0.05, 0.025, and 0.0125 mg mL\(^{-1}\) for incubation times of 24 h, 2 h, and 30 min at a temperature of 37°C and 2 h at a temperature of 4°C. Following incubation, cells were rinsed three times with cold Dulbecco’s phosphate-buffered saline (DPBS, Sigma–Aldrich) prior to scraping to form a cell suspension. The cell suspension was centrifuged, the supernatant discarded, and the pellet was resuspended in FACS buffer (1% FBS in DPBS). The samples were stored in darkness at 4°C before measurement. The results are reported in two ways: as the average percent of cells (taken over a large number of cells, typically 10 000) containing fluorescently labeled NBD-NPs and as the mean normalized fluorescence intensity of the sample. The fluorescence intensity is normalized from zero to one with respect to the maximum and the minimum (maximum for the 0.05 mg mL\(^{-1}\) 24 h sample, minimum for the blank). All results are reported as the average plus/minus the standard deviation (\(n = 6\) from two replicates in three independent experiments).

2.5.2. Confocal Microscopy

Cover slips (18 mm round, 1.5 thickness) were acid washed overnight with 1 N HCl at 60°C, rinsed with ethanol/water mixtures with successively increasing volume ratios of ethanol, and then the cover slips were placed in a 12-well plate and UV-sterilized. RAW264.7 cells were plated on the glass cover slips at 80 000 cells per well. Cells were incubated for 40 h prior to 2 h incubation with 0.05 mg mL\(^{-1}\) polycationic nanoparticles. Following incubation, cells were rinsed three times with 1× PBS pH 7.4 to remove non-internalized nanoparticles. Cells were fixed with 4% para formaldehyde in 1× DPBS for 10 min at room temperature prior to washing three times with HBSS (BioWhittaker). Cells were stained with 1 mg mL\(^{-1}\) wheat germ agglutinin (WGA) Alexa Fluor 594 conjugate for 10 min at room temperature. Cells were rinsed twice with cold HBSS and once with DI water (autoclaved). Cover slips were mounted to glass slides using Prolong Gold anti-fade reagent with DAPI (Life Technologies) and stored in the freezer prior to imaging.

Confocal images were acquired using a Zeiss LSM 710 confocal microscope with an oil immersion 63× objective. The settings for the green laser were adjusted such that blank cells (no nanoparticles) demonstrated negligible fluorescence. The step size was maintained at 0.8 mm, while the total stack volume was varied to match the cell thickness for each field. Images were collected in 16 bit format, and all images underwent identical post-processing (\(\gamma = 0.45\) for red, blue, and green channels, \(\gamma = 1.3\) for brightfield, and brightfield scale adjusted to max/min using ZEN blue).

2.5.3. Transfection with AllStars Death siRNA

RAW264.7 cells were seeded at 10 000 cells per well in 96-well plates (Nunc, Thermo Scientific). After 18 h incubation, the media was replaced with Opti-MEM. The nanoparticles were combined with siRNA (AllStars Mm/Rn Cell Death or Negative Control siRNA, Qiagen) in 1× PBS pH 5.5 at various concentrations of polymer nanoparticles and siRNA, and the complexes were added to the Opti-MEM in the wells following 1 h of incubation. For Lipofectamine containing wells, the transfection agent was complexed with the siRNA as directed (Life Technologies). The complexes were added to cells that had incubated for 1 h with Opti-MEM. The final concentration of Lipofectamine was 0.25 L well\(^{-1}\). The cells were incubated undisturbed with the siRNA/carrier in Opti-MEM for 48 h. Following incubation, media was removed and cells were incubated for 90 min with CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS, Promega) with serum-free DMEM without phenol red (Sigma–Aldrich). The absorbance at 690 nm (background) and 490 nm (MTS assay) was measured using a microplate reader (Synergy HT, BioTek Instruments, Inc.), and cell viability (\(V\)) was calculated as shown in Equation 2:

\[
V = \frac{A_{490\text{ sample}} - A_{690\text{ sample}} - (A_{490\text{ death control}} - A_{690\text{ death control}})}{A_{490\text{ live control}} - A_{690\text{ live control}} - (A_{490\text{ death control}} - A_{690\text{ death control}})}
\]

(2)

The results for viability for the AllStars death and the scrambled siRNA were compared by Student’s t-test (two-tailed, unequal variance) to check for statistically significant knockdown within each formulation (rather than comparing formulations). The percent knockdown was calculated from the cell viability for cells transfected with AllStars death siRNA and scrambled (negative control) siRNA as shown in Equation 3:

\[
\%\text{ Knockdown} = 100 \times \left(1 - \frac{V_{\text{death}}}{V_{\text{scrambled}}}\right)
\]

(3)

3. Results and Discussion

The polycationic nanoparticles were synthesized using ARGET ATRP. The interactions between polycationic nanoparticles and siRNA were analyzed using a fluorescent RiboGreen assay and using dynamic light scattering. The interactions between polycationic nanoparticles and murine macrophage cells were analyzed using flow cytometry.
and confocal microscopy. Polycationic nanoparticles loaded with siRNA were used to transfect RAW264.7 cells in order to examine the dependence of knockdown efficiency on the concentration of polycationic nanoparticles and siRNA.

3.1. Binding of siRNA

The binding of siRNA was determined from measurements of free siRNA taken using a RiboGreen RNA Assay Kit for a range of polymer to siRNA mass ratios of 80–0.0049 (see Figure 2). For all measurements, the siRNA concentration was maintained at 100 nM as the polymer nanoparticle concentration was varied. When the mass ratio of polymer to siRNA decreases below 1, the binding efficiency decreases sharply. The binding efficiency continues to decrease as the mass ratio of polymer to siRNA decreases until it begins to level off (reaching 14% binding) near a mass ratio of polymer to siRNA of 0.0195. This binding curve indicates that the polycationic nanoparticles efficiently bind siRNA at mass ratios of polymer/siRNA greater than 1. For mass ratios less than one, the polycationic nanoparticles quickly reach their maximum binding capacity and the binding efficiency decreases sharply.

3.2. Dynamic Light Scattering

*z*-Average diameter, polydispersity, and zeta potential were measured using dynamic light scattering with a Malvern ZetaSizer NanoZS instrument (see Table 3). Samples were prepared with a final concentration of 0.0125 mg mL⁻¹ polymer nanoparticles in 0.1× PBS pH 5.5 with varying concentrations of siRNA. The diameter based on the main intensity peak ranged from 260 nm (mass ratio 80) to 200 nm (mass ratio 5), while the *z*-average diameter ranged from 195 nm (mass ratio 80) to 171 (mass ratio 1.25). The zeta potential was indistinguishable for the five concentrations of siRNA with 0.025 mg mL⁻¹ polycationic nanoparticles tested (24.2–25.5 mV).

3.3. Flow Cytometry to Evaluate Uptake of Polycationic Nanoparticles

The uptake of fluorescently labeled polycationic nanoparticles was evaluated using flow cytometry (see Figure 3). The percent of cells with nanoparticles increased as the incubation time increased, indicating uptake continues after 2 h. The percent of cells with nanoparticles also increased as the concentration of nanoparticles increased, although the difference was slight for the 2 h and 30 min incubation times. The maximum percent of cells containing fluorescently labeled nanoparticles was 54% for 0.05 mg mL⁻¹ at 24 h. Likewise, the normalized.

![Figure 2. Binding curve of siRNA electrostatic binding to polycationic nanoparticles. Free siRNA quantified using a RiboGreen RNA assay kit (fluorescence-based detection). The concentration of siRNA was maintained at 100 nM and the concentration of polycationic nanoparticles was varied to achieve a range of polymer to siRNA ratios.](image-url)

<table>
<thead>
<tr>
<th>Polymer/siRNA ratio [g g⁻¹]</th>
<th>siRNA concentration [nM]</th>
<th><em>z</em>-Average diameter [nm]</th>
<th>Mean diameter, intensity</th>
<th>Zeta potential [mV]</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>0</td>
<td>190</td>
<td>261</td>
<td>25.5</td>
<td>0.270</td>
</tr>
<tr>
<td>80</td>
<td>12.5</td>
<td>195</td>
<td>256</td>
<td>25.5</td>
<td>0.277</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>194</td>
<td>236</td>
<td>25.2</td>
<td>0.262</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>176</td>
<td>201</td>
<td>24.2</td>
<td>0.262</td>
</tr>
<tr>
<td>1.25</td>
<td>800</td>
<td>171</td>
<td>222</td>
<td>25.1</td>
<td>0.241</td>
</tr>
</tbody>
</table>

Samples (unfiltered) were prepared in 0.1× PBS pH 5.5 with 0.0125 mg mL⁻¹ polymer nanoparticles (unlabeled) and varying concentrations of siRNA.
fluorescence intensity increased as the incubation time and nanoparticle concentration increased. Negligible uptake was observed at 30 min (only 6, 6, and 4% for 0.05, 0.025, and 0.0125 mg mL\(^{-1}\), respectively).

The NBD-NPs demonstrate a temperature-dependent uptake profile; incubation at 37°C (vs. 4°C) results in an increase in the percent of cells that have taken-up NBD-NPs (see Figure 4). When comparing the percent of cells with fluorescently labeled nanoparticles, there is a statistically significant difference in the uptake observed with 0.05 and 0.025 mg mL\(^{-1}\) at 37°C versus 4°C (\(p = 0.0006\) and \(p = 0.007\), respectively, using Student’s \(t\)-test with two tails and unequal variance). The reduced uptake observed at 4°C is consistent with nanoparticle internalization that is dominated by active transport processes (endocytosis) rather than by a method where the cells were passive recipients of membrane-disruption by the nanoparticles.

### 3.4. Confocal Microscopy to Verify Internalization of Polycationic Nanoparticles

The internalization of nanoparticles was confirmed using confocal microscopy. Confocal images were acquired with a Zeiss LSM 710 confocal microscope with a 63× objective. Z-stacks were used to gain a more complete 3D picture of the cells (see the orthogonal view in Figure 5) in addition to 2D snapshots (see Figure 6). The confocal images show green fluorescently labeled nanoparticles internalized within the red WGA Alexa Fluor 594 conjugate-stained membrane. Cells without green fluorescently labeled nanoparticles show negligible green fluorescence (see Supporting Information).
3.5. Transfection with AllStars Death siRNA

The dependence of knockdown on the concentration of polycationic nanoparticles and siRNA was determined by transfection of RAW264.7 cells with AllStars Death siRNA followed by an MTS assay to evaluate viability. RAW264.7 cells were transfected with 0.05, 0.025, 0.0125, and 0 mg mL\(^{-1}\) polycationic nanoparticles with 200, 100, 50, 10, and 0 nM siRNA (AllStars death or scrambled). For the two higher concentrations of polycationic nanoparticles (0.05 and 0.025 mg mL\(^{-1}\)), knockdown was observed for all four (non-zero) concentrations of siRNA (see Figure 7). Higher concentrations of nanoparticles and siRNA was associated with increased knockdown.

Knockdown was also observed with 0.0125 mg mL\(^{-1}\) polycationic nanoparticles at the siRNA concentrations of 200, 100, and 50 nM (32, 35, and 34%, respectively) (see Figure 7). Surprisingly, a small but statistically significant percentage of knockdown (<20% knockdown, \(p < 0.02\)) was observed for the highest concentration (200 nM) of naked siRNA. Although surprising because naked siRNA is not efficiently taken-up by cells, early siRNA experiments and clinical trial attempts used naked siRNA without a carrier vehicle and saw sufficient knockdown to motivate further studies.\(^{[39]}\) Transfection with Lipofectamine/siRNA complexes failed to result in statistically significant knockdown; the polycationic nanoparticles outperformed the commercially available transfection agent Lipofectamine under the experimental conditions tested. As observed in Figure 8, there is a trade-off between transfection efficiency and viability. In Figure 8, the viability was evaluated for cells treated with
scrambled siRNA, and the absorbance determined using an MTS assay was normalized as shown in Equation 2 to determine the relative viability. The trade-off result has been observed previously using the polycationic nanoparticles with other cell types\cite{40} as well as with other transfection agents in the literature.\cite{41,42} This trade-off makes dosage optimization of the transfection agent and the siRNA particularly challenging because it suggests the “optimal” transfection efficiency will be achieved at the dose that causes the greatest “acceptable” toxicity.

For applications where increased toxicity may be acceptable (such as delivery targeted to cancer cells), increased dosage will result in increased knockdown.

4. Conclusions

A polycationic nanoparticle siRNA carrier was investigated for siRNA delivery to RAW264.7 cells. The polycationic nanoparticle efficiently bound siRNA for polymer/siRNA...
mass ratios less than 1. Dynamic light light scattering indicated that siRNA binding was associated with a decreased diameter but no significant change to the zeta potential. Internalization of fluorescently labeled nanoparticles was confirmed using confocal microscopy, and flow cytometry indicated that nanoparticle uptake increased with nanoparticle concentration and with incubation time. Knockdown experiments using AllStars death siRNA and RAW264.7 cells demonstrated increasing knockdown efficiency for higher concentrations of polycationic nanoparticles and siRNA, and this proof of knockdown may motivate additional studies of this siRNA delivery vehicle.

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