ARTICLE

pubs.acs.org/Biomac

Synthesis of Biocompatible PEG-Based Star Polymers with Cationic and Degradable Core for siRNA Delivery

Hong Y. Cho,[†,‡] Abiraman Srinivasan,[†,§] Joanna Hong,[§] Eric Hsu,[§] Shiguang Liu,[§] Arun Shrivats,[§] Dan Kwak,[§] Andrew K. Bohaty,[‡] Hyun-jong Paik,[∥] Jeffrey O. Hollinger,[§] and Krzysztof Matyjaszewski*[,‡]

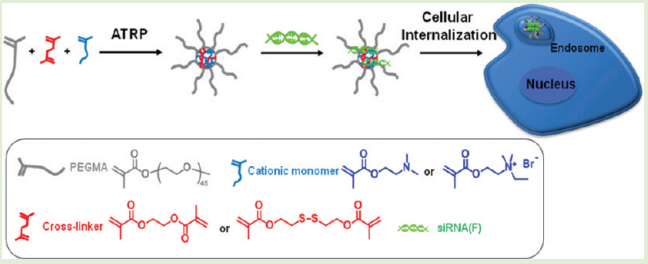
[‡]Department of Chemistry, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213, United States

[§]Bone Tissue Engineering Center, Carnegie Mellon University, 5000 Forbes Avenue, Pittsburgh, Pennsylvania 15213, United States

[∥]Department of Polymer Science and Engineering, Pusan National University, Busan, South Korea

Supporting Information

ABSTRACT: Star polymers with poly(ethylene glycol) (PEG) arms and a degradable cationic core were synthesized by the atom transfer radical copolymerization (ATRP) of poly(ethylene glycol) methyl ether methacrylate macromonomer (PEGMA), 2-(dimethylamino)ethyl methacrylate (DMAEMA), and a disulfide dimethacrylate (cross-linker, SS) via an "*arm-first*" approach. The star polymers had a diameter ~15 nm and were degraded under redox conditions by glutathione treatment into individual polymeric chains due to cleavage of the disulfide cross-linker, as confirmed by dynamic light scattering. The star polymers were cultured with mouse calvarial preosteoblast-like cells, embryonic day 1, subclone 4 (MC3T3-E1.4) to determine biocompatibility. Data suggest star polymers were biocompatible, with ≥80% cell viability after 48 h of incubation even at high concentration (800 μg/mL). Zeta potential values varied with N/P ratio confirming complexation with siRNA. Successful cellular uptake of the star polymers in MC3T3-E1.4 cells was observed by confocal microscopy and flow cytometry after 24 h of incubation.



1. INTRODUCTION

Short interfering ribonucleic acid (siRNA), also known as small interfering RNA or silencing RNA, was first discovered by Fire[1] and Baulcombe in the late 1990s.[2] An siRNA platform provides a compelling suite of options to treat cancer,[3] infectious diseases,[4] and metabolic diseases[5] as well as genetic disorders.[6] The biological reason for this approach is based on the siRNA efficiency to selectively degrade targeted messenger ribonucleic acid (mRNA) and thus silence putative genes association.[7,8] RNA interference (RNAi) plays a central role in controlling gene expression naturally in all eukaryotic cells[9] and consequently may reduce the function of an individual gene, or group of genes, without eliciting a toxic or an immune response.[10] However, "naked" siRNA cannot enter the plasma membrane due to electrostatic repulsion between the anionic plasma membrane and the anionic phosphodiester backbone of siRNA.[3,11] Furthermore, "naked" siRNAs are unstable under physiological conditions. They are susceptible to ribonucleases and are rapidly excreted via kidney and nonspecifically absorbed through the reticuloendothelial system.[12] Therefore, an efficient delivery system is required for protection, transportation, and release of siRNA inside the cell.

The two most commonly used delivery approaches for either genes or siRNA are viral and nonviral systems. Each approach has advantages and limitations. Viral delivery systems possess high efficiency in siRNA transfer, but are difficult to produce; they are toxic[13] and interfere with the host genome.[14] These limitations favor nonviral vectors. Nonviral delivery systems include peptides,[15] lipids,[16] dendrimers,[17] polymers with cationic charges,[18,19] calcium phosphate,[20] or carbon and gold nanoparticles.[21] Cationic polymers offer high affinity binding of nucleic acids and efficient delivery into cells[22] via adsorptive endocytosis.[23] These nonviral delivery systems are easy to produce, they are safe, stable, and can be synthesized in different sizes and shapes as well as contain different functionalities for targeted delivery. Despite remarkable progress in polymeric siRNA delivery system engineering, a number of limitations preclude enthusiasm including low transfection efficiency,[24] cytotoxicity,[25] and the ill-defined structure of materials obtained by conventional radical polymerization processes.[26] In the past decade, progress in controlled radical polymerizations (CRPs) has provided polymers with narrow molecular weight distributions[27,28] and used to prepare polymers for siRNA delivery.[29−33] Recently, atom transfer radical polymerization (ATRP),[34−39] one of CRP procedures, has offered excellent control over polymer structure, uniformity, topology, and functionality. Consequently, a profound new opportunity is available that provides a robust and versatile approach to tailor-made delivery vehicles for either gene or siRNA delivery into cells.[39−43]

Received: May 11, 2011
Revised: August 15, 2011
Published: September 06, 2011

Star polymers have been exploited for the design and delivery of nucleic acids,[44−48] peptide,[49] and drugs.[50] Star polymers are three-dimensional globular[51] or branched polymers containing multiple arms connected to a central core that have unique properties compared to their linear analogs.[52,53] The synthesis of star polymers is simple and cost-effective[54] and, consequently, provides a better alternative to dendrimer-based delivery systems.[55,56] Recently, we reported the synthesis of biocompatible multifunctional poly(ethylene glycol) (PEG) star polymers prepared by ATRP, which exhibited rapid cellular internalization.[57] To dissociate and release nucleic acids into cells, biodegradable stars can be prepared to release nucleic acids in response to external stimuli such as pH[58] and enzymes[59] or by selective incorporation of a reductively[60−62] or hydrolytically degradable chemical bonds.[63]

In this study, we discuss the synthesis of biocompatible star polymers with PEG arms with core structures incorporating cationic and biodegradable functionalities by ATRP via an "*arm-first*" approach[64] for siRNA delivery and cellular internalization of star polymers complexed with siRNA. The "*arm-first*" approach generates the core of the star macromolecule by coupling monofunctional "*living*" polymeric chains with a divinyl reagent. Star polymers were prepared with cationic and degradable cores through the addition of 2-(dimethylamino)ethyl methacrylate (DMAEMA) and bis(2-methacryloyloxyethyl) disulfide cross-linker (SS) to an "*arm-first*" synthesis of PEG based stars. The cationic star polymers were tested for biocompatibility, cell proliferation and cellular internalization using mouse calvarial pre-osteoblast like cells, embryonic day 1, subclone 4 (MC3T3-E1.4) cells. Further, we studied the biodegradation of star polymers prepared with SS in the presence of glutathione (GSH). The obtained data suggest the synthesized cationic star polymers offer significant improvement in biocompatibility, siRNA protection, and cellular internalization, thereby providing compelling new opportunities for effective and efficient delivery of genes and siRNA.

2. EXPERIMENTAL SECTION

2.1. Materials. PEGMA (M_n = 2080, 50 wt % in water) was extracted with methylene chloride. The organic solution was stirred with Na_2SO_4 to remove residual water before passage through a basic alumina column. The cleavable cross-linking agent bis(2-methacryloyloxyethyl) disulfide (SS) was synthesized via a previous published procedure.[65,66] CuCl (98%, Acros) was purified by stirring with acetic acid three times and washed with ethyl alcohol before being dried for 12 h at 60 °C. 2-(Dimethylamino)ethyl methacrylate (DMAEMA, 97%, Aldrich) and ethylene glycol dimethacrylate (EGDMA, 98%, Aldrich) were passed through the basic alumina to remove inhibitor. All other chemicals, ethyl 2-bromoisobutyrate (EBiB, 99%, Aldrich), bromoethane (98%, Aldrich), 1,1,4,7,10,10-hexamethyl triethylenetetramine (HMTETA, 97%, Aldrich), and solvents were used as received without further purification.

2.2. Instruments. Star polymer samples were separated by GPC (Polymer Standards Services (PSS) columns (guard, 10^5, 10^3, and 10^2 Å) with THF eluent at 35 °C, flow rate = 1.00 mL/min and differential refractive index (RI) detector (Waters 2414)). The apparent molecular weights (M_n) and polydispersities (M_w/M_n) were determined with a calibration based on linear polystyrene standards using WinGPC 7.0 software from PSS. The detectors employed to measure the absolute molecular weights ($M_{w,MALLS}$) were a triple detector system containing RI detector (Wyatt Technology, Optilab REX), viscometer detector (Wyatt Technology, ViscoStar), and a multiangle laser light scattering (MALLS) detector (Wyatt Technology, DAWN EOS) with the light wavelength at 690 nm. Absolute molecular weights were determined using ASTRA software from Wyatt Technology. ^{1}H NMR spectra of the polymers were collected on Bruker Avance 300 MHz spectrometer at 25 °C. Both particle size, size distribution, and zeta potential of star polymers were determined by using a Zetasizer Nano from Malvern Instruments, Ltd. Fluorescence microscopy was performed for cell viability using a Zeiss Axiovert 200 microscope. Confocal imaging was done on an Olympus FV1000 microscope and flow cytometry was performed using a Coulter Epix Elite flow cytometer for cellular internalization.

2.3. Synthesis and Characterization of Quaternized DMAEMA with Ethyl Bromide (QDMAEMA). DMAEMA (20.0 mL, 59.3 mmol) containing polymerization inhibitor was dissolved in 40.0 mL of acetone. The solution was cooled in an ice−water bath, and to the stirring solution bromoethane (23.0 mL, 118.6 mmol) was added dropwise under stirring over a period of 30 min. The mixture was stirred for an additional 24 h at room temperature, followed by dried in vacuo and precipitated against cold ethyl ether. Yield: 13.8 g (87.3%). ^{1}H NMR (δ, ppm, in $CDCl_3$): 6.13 (s, 1H, =CH) 5.67 (s, 1H, =CH), 4.65 (m, 2H, CH_2O), 4.14 (m, 2H, CH_2N^+), 3.79 (m, 2H, CH_2C), 3.50 (s, 6H, $(CH_3)_3N^+$), 1.94 (s, 3H, $=CCH_3$), and 1.45 (t, 3H, CCH_3).

2.4. Synthesis of Star Polymers with PEG Arms and with a Cationic Degradable Core. $(PEG)_{50}$-poly$(DMAEMA_{22}$-*co*-$SS_{46})$ star polymers were synthesized by ATRP via an "*arm-first*" method. A typical procedure is briefly described. The ratio of reagents used is $[PEGMA]_0/[DMAEMA]_0/[SS]_0/[EBiB]_0/[CuCl/HMTETA]_0$ = 1/0.5/1/0.21/0.42. PEGMA (M_n = 2080, 2.0 g, 1.0 mmol), DMAEMA (78.6 mg, 0.5 mmol), SS (290.4 mg, 1.0 mmol), HMTETA (114.2 μL, 0.42 mmol), and methanol (20 mL) were charged to a Schlenk flask. The flask was degassed by five freeze−pump−thaw cycles and filled with nitrogen. CuCl (42.0 mg, 0.42 mmol) was quickly added to the frozen mixture under nitrogen. The flask was sealed with a glass stopper then evacuated and backfilled with nitrogen five times before being immersed in a 60 °C oil bath. The deoxygenated initiator EBiB (31.0 μL, 0.21 mmol) was injected into the reaction system, via a nitrogen-purged syringe, through the side arm of the Schlenk flask with the frozen solution was thawed. At timed intervals, samples were withdrawn via a syringe fitted with stainless steel needle and immediately diluted with acetone to follow conversion by gas chromatography (GC) analysis. The samples were used to measure polymer molecular weights by GPC in THF. The reaction was stopped after 72 h by exposure to air. The final star polymers were purified by dialysis against methanol and distilled water for 2 days, respectively, by using a dialysis bag with MWCO = 25000. Molecular weight of star polymer was determined by MALLS and overall star composition by GC conversion, resulting in a star with 50 PEGMA arms and a core consisting of 22 DMAEMA unit and 46 SS cross-linker units, corresponding to $(PEG)_{50}$-poly$(DMAEMA_{22}$-*co*-$SS_{46})$.

$(PEG)_{51}$-poly$(QDMAEMA_{22}$-*co*-$SS_{45})$ was synthesized and characterized in a similar way using following ratio for the reagents $[PEGMA]_0/[QDMAEMA]_0/[SS]_0/[EBiB]_0/[CuCl/HMTETA]_0$ = 1/0.5/1/0.21/0.42. For the synthesis, PEGMA (M_n = 2080, 2.0 g, 1.0 mmol), QDMAEMA (133.1 mg, 0.5 mmol), SS (290.4 mg, 1.0 mmol), HMTETA (114.2 μL, 0.42 mmol), CuCl (42.0 mg, 0.42 mmol), and EBiB (31.0 μL, 0.21 mmol) were used. For the control degradation experiment, nondegradable $(PEG)_{53}$-poly$(DMAEMA_{22}$-*co*-$EGDMA_{45})$ and noncationic $(PEG)_{43}$-polySS_{45} star polymers were prepared under the same reaction conditions. After purification by dialysis, star polymers were analyzed for copper residue using inductively coupled plasma mass spectrometry (ICP-MS, ACS Laboratories, TX).

2.5. Degradation of Star Polymers in Glutathione Solution. Star polymers were dissolved in DNAase/RNAase free-distilled water without any additional surfactants and mixed with a glutathione (GSH) solution. The final PEG star and GSH concentration was 2 mg/mL and 100 mM (supraphysiological concentration), respectively. The solution was incubated with 5% CO_2 at 37 °C for 48 h. Degradation experiments were also conducted under a physiological concentration, where 0.25 mg/mL of each star polymer was degraded under 10 mM

GSH solution under N_2 at room temperature for 4 days. The variation in the hydrodynamic diameter of $(PEG)_{50}$-poly($DMAEMA_{22}$-*co*-SS_{46}), $(PEG)_{51}$-poly($QDMAEMA_{22}$-*co*-SS_{45}), and $(PEG)_{43}$-polySS_{45} was measured by dynamic light scattering (DLS).

2.6. Cell Culture. Mouse calvarial preosteoblast-like cells embryonic day1, subclone 4 (MC3T3-E1.4) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in α-minimum essential medium (α-MEM), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). Cell culture flasks and culture plates with flat bottoms (Corning) and 96-well suspension culture plates with U-shaped bottoms (Greiner) were used for suspension culture. Trypsin-ethylenediaminetetraacetic acid (EDTA) for cell passaging and a live/dead viability/cytotoxicity kit were purchased from Invitrogen. N-TER, a commercially available peptide-based delivery system was obtained from Sigma used as a positive control for cell internalization study.

2.7. Cell Viability Assay. $(PEG)_{50}$-poly($DMAEMA_{22}$-*co*-SS_{46}) and $(PEG)_{51}$-poly($QDMAEMA_{22}$-*co*-SS_{45}) star polymers were tested for cell viability using the live/dead cytotoxicity assay kit. A total of 5×10^4 MC3T3-E1.4 cells per well were seeded in a 24-well tissue culture polystyrene plate containing 900 μL of α-MEM media and 100 μL of sample of respective star polymers (200 μg/mL) was added after sterile filtration using 0.22 μm syringe filter. After 24 h incubation of star polymers with cells, the cell culture media was aspirated and washed with PBS. Thereafter, 0.5 mL of live/dead stain was added (calcein 1:2000 and ethidium homodimer 1:500, diluted in PBS) to the cells and incubated at 37 °C for 30 min in the dark and images were captured using a Zeiss Axiovert 200 fluorescence microscope.

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay was performed to determine the cell viability quantitatively, using a commercially available kit (CellTiter 96 aqueous one solution assay Promega G3580). Briefly, MC3T3-E1.4 cells were seeded at a density of 10000 cells/mL in a 48-well plate (Costar 3548) and cultured for 48 h. Exposure to star polymers was initiated once the cells had reached 80% confluency. Cells alone were used as a negative control (no decrease in cell viability expected). For the experimental groups, media containing concentrations of 100, 200, 400, and 800 μg/mL of star polymers were added. Cells with the same range of concentration of polyethyleneimine (PEI; Sigma 408727) were used as a positive control (expected decrease in cell viability with increased PEI concentration). After culturing for an additional 48 h, media was collected in 1.5 mL eppendorf tubes (Fisherbrand 02-681-284), and 200 μL of that returned to its respective well on the 48-well plate. The plates were then incubated at 37 °C and 5% CO_2 for another 60 min to allow the pH of the media to stabilize. Once completed, 40 μL of MTS reagent was added to each well and incubated at 37 °C and 5% CO_2 for 20−30 min. The absorbance was recorded at 492 nm using a Tecan Spectra Fluor. The percentage of cell viability was normalized to cells alone, which was set to 100% cell viability. Percentage cell viability was calculated using the following formula:

$$\%\text{cellviability} = \frac{OD_{sample} - OD_{media}}{OD_{cellls} - OD_{media}}$$

2.8. Cell Proliferation. Cells were cultured in 12-well tissue culture plates (Costar 3513) for MC3T3-E1.4 cells over a period of 10 days. Polymers were added (100 μg/mL of star polymers and 1 μg/mL of PEI) to cells, 48 h after seeding, and incubated at 37 °C and 5% CO_2. The media was changed every 48 h, with the same concentration of polymers added to the new media each time. Samples were collected at 0, 4, 7, and 10 days of treatment. At each time period, the media was removed and 600 μL of 1× cell lysis buffer (Cell Signaling 9803) added to each of the wells. The plates immediately went through three freeze−thaw cycles (each cycle involves incubation at −80 °C for 20 min, followed by incubation at 37 °C for 20 min). Samples were transferred to 1.5 mL eppendorf tubes (Fisherbrand 02-681-284) and centrifuged at 13000 rpm for 7 min to remove debris. The contents of each tube were then transferred to two eppendorf tubes and frozen at −80 °C until ready for use. The final volume of PicoGreen solution needed was determined by calculating the total number of wells to be used. Concentrated PicoGreen was then diluted 200-fold to form the final volume needed. A standard DNA curve was made by diluting concentrated DNA standard with 1× cell lysis buffer (Cell Signaling 9803) to form what would become concentrations of 0, 31.25, 62.5, 125, 250, 500, and 1000 ng/mL of DNA. Experimental samples were thawed at room temperature. Once this was completed, 100 μL of each DNA standard, as well as the experimental samples were added to a 96-well plate (Fisherbrand 12-565-501). Following this, 100 μL of diluted PicoGreen solution was added to each well and incubated for 3 min. The fluorescence was then recorded at fluorescein excitation (485 nm) and emission (535 nm) wavelengths using a Tecan Spectra Fluor. A standard curve was created with the OD values of the DNA standards and a linear correlation determined between OD value and DNA concentration.

2.9. Zeta Potential and Size Distribution Measurement. Star polymers/siRNA complexes were prepared at various N/P molar ratios from 0.2:1 to 10:1 by adding various amounts of siRNA (negative control siRNA, Ambion, TX) with 50 μM concentration. Complexation was carried at 4 °C for 2 h and then incubated for 30 min at room temperature. Zeta potential and size distribution of each sample were determined using three repeats, each repeat was measured three times using a Zetasizer Nano (Malvern Instruments, U.K.).

2.10. Polyion Complex of siRNA and Star Polymers for Confocal Microscopy and Flow Cytometry. Fluorescein isothiocyanate (FITC) conjugated siRNA(F) and cyanine 3 (Cy3) conjugated siRNA(Cy3) were obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). siRNA was resuspended to form 125 μL of stock siRNA solution and then diluted with 875 μL of sterile, nuclease-free water to form a final concentration of 2.5 mM siRNA. This solution was then added to dry star polymers (2 mg) and mixed overnight at 4 °C. Briefly, 22.6 nmol of $(PEG)_{50}$-poly($DMAEMA_{22}$-*co*-SS_{46}) with 2.5 μmol of siRNA and 22.2 nmol of $(PEG)_{51}$-poly($QDMAEMA_{22}$-*co*-SS_{45}) with 2.5 μmol of siRNA were incubated, respectively. Using a dialysis membrane (Spectra/Por Biotech Regenerated Cellulose Dialysis Membranes) with MWCO = 50000, the siRNA-star polymer polyion complex was separated from unbound siRNA after 24 h dialysis at 4 °C.

2.11. Cellular Internalization by Confocal Microscopy. A total of 5×10^4 of MC3T3-E1.4 cells were seeded directly onto a 96-well U-shaped bottom, nonadherent tissue culture plate. The cells were incubated with 200 μg/mL of siRNA conjugated star polymers at 37 °C for 24 h. The control group did not receive any polymers. After 24 h, the media was aspirated and the cells were washed in PBS and trypsinized and seeded onto sterile Lab-Tek II chambered coverglass. After overnight attachment, the cells were washed with PBS and were fixed with 4% paraformaldehyde for 10 min, and examined by confocal microscopy (Olympus FV1000 microscope). All imaging conditions for the confocal microscopy, including laser power, photomultiplier tube, and offset settings, remained constant for each comparison set.

2.12. Cellular Internalization by Flow Cytometry. A total of 5×10^5 MC3T3-E1.4 cells were suspended directly onto a 96-well U-shaped bottom nonadherent tissue culture plate and incubated with ~200 μg/mL of siRNA conjugated star polymers for 24 h at 37 °C. The control group did not receive any star polymers. After 24 h, the media was aspirated and cells were washed in PBS and trypsinized then collected into 1.5 mL centrifuge tubes and centrifuged at 2000 rpm for 3 min to pellet the cells. The supernatant was removed and the cells were resuspended in 1 mL of sterile PBS and transferred to a borosilicate tube. Each sample was analyzed using a Coulter Epix Elite Flow cytometer.

Scheme 1. Synthesis of Star Polymers with PEG Arms and with Cationic and Degradable Core by ATRP via an "*Arm-First*" Method and Cell Internalization of Star Polymers Complexed with siRNA

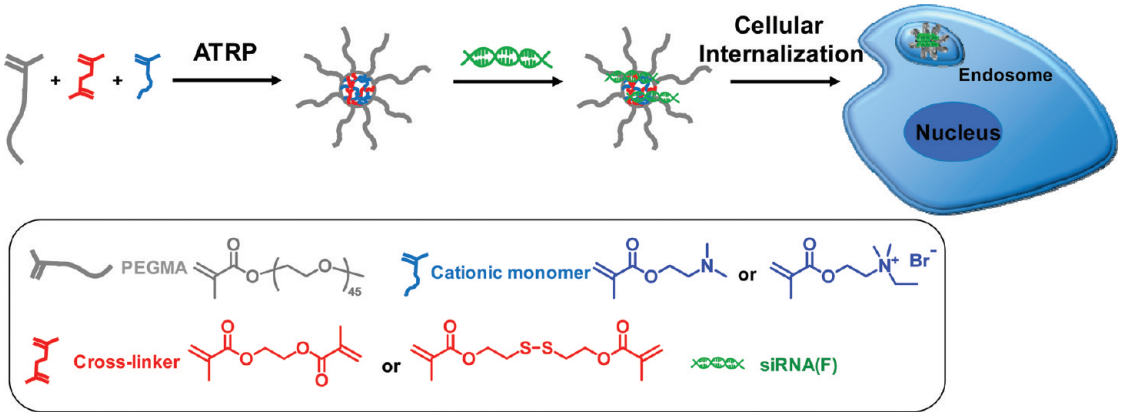


Table 1. Poly(ethylene glycol) Based Star Polymers by ATRP via an "*Arm-First*" Method[a]

entry	cross-linker	$M_{n,RI}$[b] $\times 10^{-3}$	M_w/M_n[c]	$M_{w,MALLS}$[d] $\times 10^{-3}$	N_{arm}[e]	D_h[f] (nm)
N-SS[g]	SS	39.9	1.33	88.8	50	10.3 ± 2.07
QN-SS[h]	SS	40.6	1.39	90.3	51	15.2 ± 1.58
N-EGDMA[i]	EGDMA	41.1	1.33	94.7	53	10.4 ± 0.90
0-SS[j]	SS	34.9	1.36	74.7	43	14.5 ± 1.68

[a] N = DMAEMA; QN = QDMAEMA; 0 = noncationic (neutral). Experimental conditions: $[PEGMA]_0 = 0.05$ M at 60 °C in methanol, stopped at 72 h. [b] Number-average molecular weight, measure by THF GPC with RI detector, calibrated with linear PS standards. [c] Polydispersity as a value of M_w/M_n, measured by THF GPC with RI detector. [d] Weight-average molecular weight, measured by THF GPC with MALLS detector. [e] Number-average value of the number of arms per star molecule. [f] Hydrodynamic volume in RNase/DNase free water, measured by DLS and size expressed as D_{avg} ± SD (average diameter ± standard deviation). [g] $[PEGMA]_0/[DMAEMA]_0/[SS]_0/[EBiB]_0/[CuCl/HMTETA]_0 = 1/0.5/1/0.21/0.42$. [h] $[PEGMA]_0/[QDMAEMA]_0/[SS]_0/[EBiB]_0/[CuCl/HMTETA]_0 = 1/0.5/1/0.21/0.42$. [i] $[PEGMA]_0/[DMAEMA]_0/[EGDMA]_0/[EBiB]_0/[CuCl/HMTETA]_0 = 1/0.5/1/0.21/0.42$. [j] $[PEGMA]_0/[SS]_0/[EBiB]_0/[CuCl/HMTETA]_0 = 1/1/0.21/0.42$.

2.13. Statistical Analysis. Means were statistically compared by one-way ANOVA through SPSS software. For cell viability and cell proliferation results, multiple comparison was carried out if the ANOVA is significant ($P < 0.05$). The single asterisk indicated the significance to control group with $P < 0.05$. The double asterisks indicated the significance to control group with $P < 0.01$. For zeta potential analysis, the Student's *t*-test was used to determine whether data groups differed significantly from each other. Statistical significance was defined as having $P < 0.001$ (denoted as three asterisks).

3. RESULTS

3.1. Synthesis and Characterization of Star Polymers. Star polymer were prepared by an "*arm-first*" method in a one-pot synthesis procedure, by copolymerization of PEGMA with DMAEMA or QDMAEMA, and SS cross-linker using an EBiB initiator and CuCl/HMTETA as catalyst (Scheme 1).[54,57] GPC analysis showed decreased intensity of PEGMA macromonomer after 72 h, suggesting effective incorporation of macromonomers into the star polymers.

Star polymer composition was calculated based on monomer, macromonomer and cross-linker conversion by GC, GPC and $M_{w,MALLS}$. The absolute molecular weights, $M_{w,MALLS}$, of $(PEG)_{50}$-poly$(DMAEMA_{22}$-*co*-$SS_{46})$ (Table 1, entry N-SS) and $(PEG)_{51}$-poly$(QDMAEMA_{22}$-*co*-$SS_{45})$ (Table 1, entry QN-SS) after purification by dialysis membrane bag (MWCO = 25000) were 88.8 × 10^3 and 90.3 × 10^3, which are higher than the apparent molecular weights, $M_{w,RI}$, 39.9 × 10^3 and 40.6 × 10^3, respectively, due to their compact structure. M_w/M_n of N-SS and QN-SS were 1.33 and 1.39, respectively. The yields of stars measured by the multipeak splitting of the GPC curves using Gaussian function were 72% for N-SS and 74% for QN-SS. N-SS have 50 PEG arms connected to the core with 22 units of DMAEMA and 46 units of SS. The hydrodynamic volume measured by DLS in DNase/RNase free water was 10.3 nm for N-SS and 15.2 nm for QN-SS, respectively. $(PEG)_{53}$-poly$(DMAEMA_{22}$-*co*-$EGDMA_{46})$ (Table 1, entry N-EGDMA, M_n = 41.1 × 10^3, M_w/M_n = 1.33) and $(PEG)_{43}$-polySS_{45} (Table 1, entry 0-SS, M_n = 34.9 × 10^3, M_w/M_n = 1.36) were prepared as a nondegradable and noncationic control samples, respectively. After purification by dialysis, single peak was observed with GPC analysis, indicating successful removal of PEGMA. The amount of residual copper in N-SS, QN-SS, and 0-SS was 23.84, 3.67, and 10.54 ppm as measured by ICP-MS analysis.

3.2. Degradation of N-SS and QD-SS. DLS analysis of the hydrodynamic diameter of N-SS and QD-SS after 48 h incubation with a supraphysiological concentration (100 mM) of GSH solution, showed decrease in particle size, suggesting that the core of the star polymers was degraded into individual polymeric chains, whereas the control samples to which 0 mM GSH was added did not show any such change (Figure 1A,B) For a negative control experiment, nondegradable N-EGDMA was tested under the same conditions of N-SS and QN-SS. The size distribution N-EGDMA with 100 mM GSH showed similar size distribution

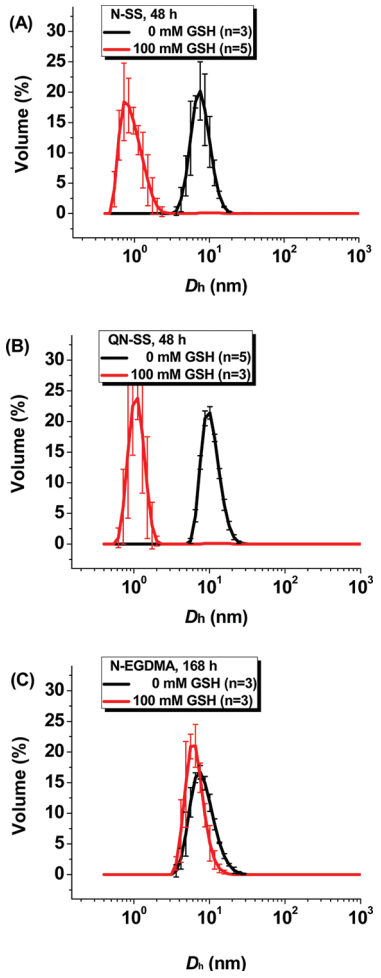


Figure 1. Size distribution of N-SS, QN-SS, and N-EGDMA star polymers after glutathione (GSH) treatment in 48 h, determined by volume % DLS analysis. (A) N-SS with 0 mM (black curve, $n = 3$) and 100 mM (red curve, $n = 5$) GSH addition. (B) QN-SS with 0 mM (black curve, $n = 3$) and 100 mM (red curve, $n = 5$) GSH addition. (C) N-EGDMA with 0 mM (black curve, $n = 3$) and 100 mM (red curve, $n = 3$) GSH addition.

to that of the control group (0 mM GSH treatment), which suggests EGDMA cross-linker is nondegradable in the presence of GSH (Figure 1C).

Degradation of star polymers was also examined under a physiological GSH concentration (10 mM) under N_2 at room temperature. DLS analysis of N-SS and QD-SS showed successful degradation by 4 days, whereas 0-SS showed complete degradation only after 6 days of incubation (Table 2).

3.3. Cell Viability. The synthesized star polymers were examined for in vitro cell viability (cytotoxicity) after thorough washing of all the residual copper catalyst from the polymers, cationic compounds, such as DMAEMA or QDMAEMA, and vinyl group moieties were evaluated for their effects on cell viability using MC3T3-E1.4 cells. After 24 h incubation, live/dead staining (Figure 2) of MC3T3-E1.4 cells showed >85% viable cells and the percentage of viable cells were comparable to the control group, which received no polymers.

MTS results for MC3T3-E1.4 after 48 h incubation of star polymers in concentrations 100, 200, 400, and 800 μg/mL are shown in Figure 3. Decreased cell viability was observed for 0-SS

Table 2. Degradation under a Physiological GSH Concentration (10 mM) Analyzed as a Hydrodynamic Diameter by DLS[a]

entry	before degradation (nm)	after degradation (nm)	time (days)
N-SS	10.3 ± 2.07	0.97 ± 0.22	4
QN-SS	15.2 ± 1.58	0.97 ± 0.15	4
0-SS	14.5. ± 1.68	1.00 ± 0.15	6

[a]Polymer concentration 0.25 mg/mL with DNase/RNase free water under N_2 at room temperature, hydrodynamic diameter (nm): mean ± S.D., $n = 3$.

at 400 μg/mL ($P < 0.05$) and 800 μg/mL ($P < 0.01$) but this trend was not observed for the N-SS and QN-SS star polymers, whereas commercially available PEI showed 60−70% decreased cell viability ($P < 0.01$) at all concentrations.

3.4. In Vitro Cell Proliferation. Picogreen assay showed stable cell proliferation for star polymers (100 μg/mL) and was comparable to that of control group for which no polymers were treated (Figure 4). However, PEI polymers (1 μg/mL) showed significant decrease in cell proliferation after 4, 7, and 10 days of incubation (Figure 4). Cell proliferation was statistically significantly ($P < 0.01$) for star polymers when compared to PEI.

3.5. Zeta Potential and Size Distribution of siRNA Complexes. Solution 1 mg/mL of star polymer under DNase/RNase free water was prepared. Aliquots of siRNA solution were added to the star polymer solution, corresponding to molar N/P ratios based on the overall star composition and siRNA. N-SS, QN-SS, and 0-SS were evaluated for complexation to siRNA by zeta potential measurements (Figure 5). Incorporation of cationic monomers DMAEMA or QDMAEMA to the star polymers facilitated electrostatic complexation with the negatively charged siRNA. The initial zeta potential of N-SS and QN-SS was 22.5 ± 1.32 and 2.38 ± 0.73 mV, while the zeta potential of 0-SS was −6.68 ± 1.90 mV because of different core charges. As shown in Figure 5, the zeta potential value of N-SS decreased from 22.9 ± 1.78 mV to −26.3 ± 1.27 mV with increasing siRNA amount (with degreasing N/P ratio from 10 to 0.2). In the case of QN-SS, the variation of zeta potential on N/P ratio is small, indicating the efficacy of siRNA complexation of N-SS is greater than for QN-SS. Student's *t*-test analysis was performed to determine statistical significance of star polymers only and siRNA complexed star polymers with different N/P ratios (N/P = 10 to 0.2). Significant difference ($P < 0.001$) was observed for N-SS from N/P = 1 to 0.2; QN-SS and 0-SS from N/P = 10 to 0.2, suggesting efficient complexation of siRNAs to star polymers at these N/P ratios. The hydrodynamic volume of the star polymer complexed with siRNA did not change significantly with N/P ratios (for example, hydrodynamic diameter of QN-SS varied from 12.53 ± 1.00 to 12.82 ± 1.00 nm for N/P = 0.2 and 10, respectively), suggesting siRNA complexed star polymers stayed as individual polymers without any aggregation.

3.6. Cellular Internalization of siRNA Complexed Star Polymers. Confocal laser microscopic analysis suggested 100% cellular internalization of siRNA(F) complexed N-SS (Figure 6C) and QN-SS (Figure 6D) after 24 h incubation. Cellular internalization results were comparable to that of commercially available N-TER. However, siRNA complexed with N-TER revealed large aggregates (Figure 6B). In contrast, the cationic star polymers (Figure 6C,D) presented a uniform, pancytoplasmic and perinuclear distribution in MC3T3-E1.4 cells. Mild fluorescence was observed for cells incubated with "naked" siRNAs (Figure 6A),

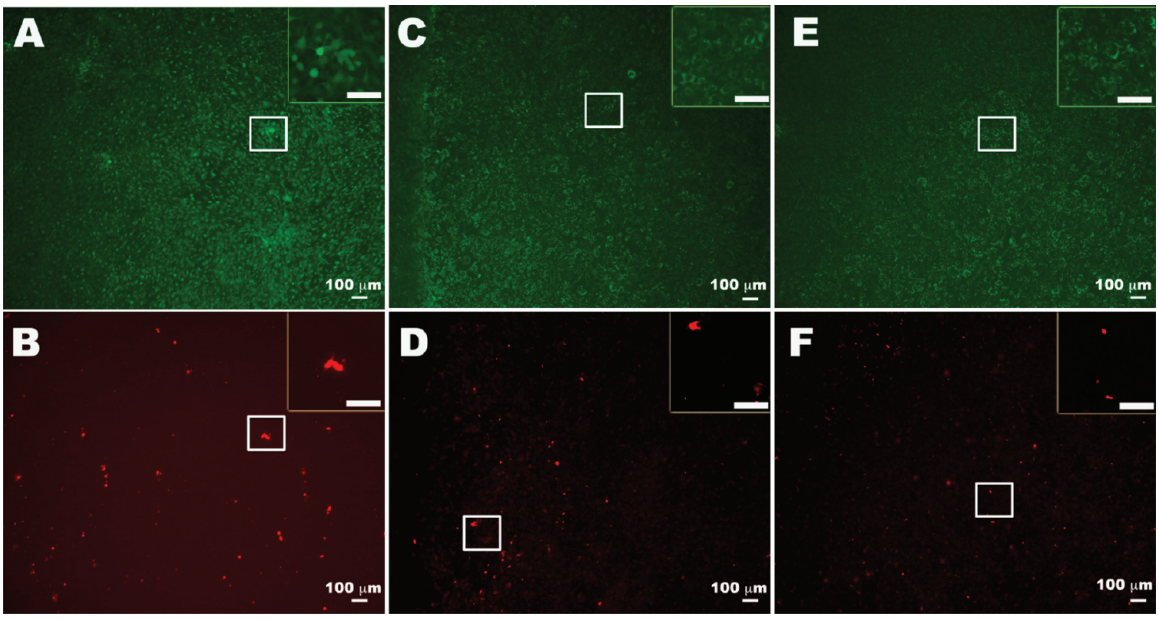


Figure 2. MC3T3-E1.4 cells after 24 h incubation with N-SS (C and D) and QN-SS (E and F) star polymers were nontoxic and the cell viability was comparable to untreated control group (A and B). Live cells were stained green (A, C, and E) and dead cells were stained red (B, D, and F). The higher magnification inset scale bar = 50 μm.

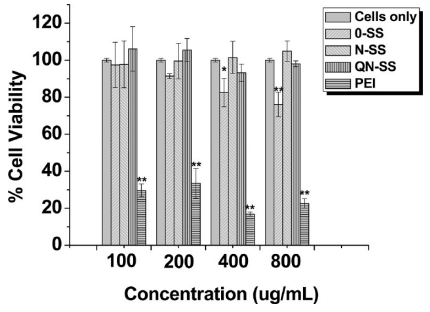


Figure 3. In vitro cell viability of MC3T3-E1.4 cells determined by MTS after 48 h. The star polymers, 0-SS, N-SS, and QN-SS, were nontoxic, and the percent cytotoxicity was comparable to cells only that did not receive any star polymers. Data are reported as mean ± S.D.; $n = 3$. Statistical significance was assessed by performing one-way analysis of variance (ANOVA) through SPSS software. Logarithmic standard deviation (LSD) test for multiple comparison was carried out if the ANOVA is significant ($^*P < 0.05$, $^{**}P < 0.01$).

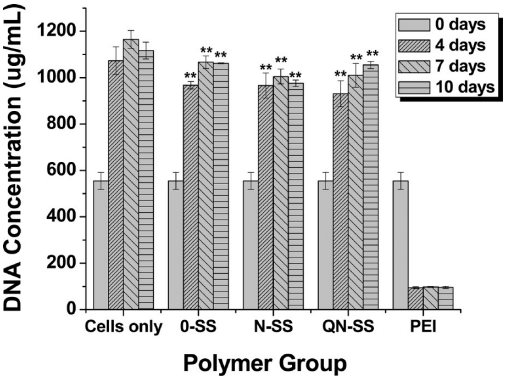


Figure 4. PicoGreen results for MC3T3-E1.4 after 10 days. The star polymers (100 μg/mL) appeared to have minimal, if any, significant effect on MC3T3-E1.4 proliferation compared to cells alone. However, PEI (1 μg/mL) fully suppressed proliferation. Data are reported as mean ± S.D.; $n = 3$. Statistical significance was assessed by performing one-way analysis of variance (ANOVA) through SPSS software. LSD test for multiple comparison was carried out if the ANOVA is significant ($^*P < 0.05$, $^{**}P < 0.01$).

signifying poor cellular internalization. Z-stack confocal microscopic image of star polymers exhibiting successful internalization in MC3T3-E1.4 cells are shown in Figure S1 and S2 (in the Supporting Information). FACS analysis (Figure 7) further confirmed cellular internalization for N-SS and QN-SS siRNA-(Cy3) complexed star polymers in MC3T3-E1.4 cells after 24 h incubation.

4. DISCUSSION

The objective of this study was to determine biocompatibility, biodegradation, siRNA binding, and in vitro cellular internalization of star polymers with PEG arms and with a cationic degradable core. DMAEMA-based systems were previously used as a potential vehicle for gene delivery, due to their cationic properties. However, the possible cytotoxicity was a key issue.[67,68] In our approach, we prepared star polymers with DMAEMA incorporated into the core protected by external PEG arms to enhance biocompatibility and retain cationic properties.[69] PEG is a biocompatible, water-soluble polymer and is widely used as a polymeric gene delivery carrier.[70] PEGylated nanocarriers offer diminished enzymatic degradation and hence, require a chemically cross-linked backbone for in vivo degradation. Consequently, one approach is to use of a disulfide cross-linker that will degrade under redox conditions.[60−62] The rationale for polymers with disulfide-functionalized cross-linkers is to produce biomaterials that undergo controlled biodegradation and thus will have predictable biological performance properties as therapeutics.[71] Moreover, the disulfide bond is naturally present in a majority of proteins and enzymes and is cleaved in vivo

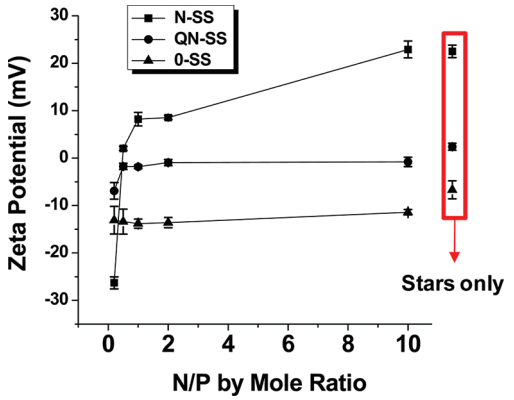


Figure 5. Zeta potential values as a function of N/P ratio for complexation of star polymer with siRNA.

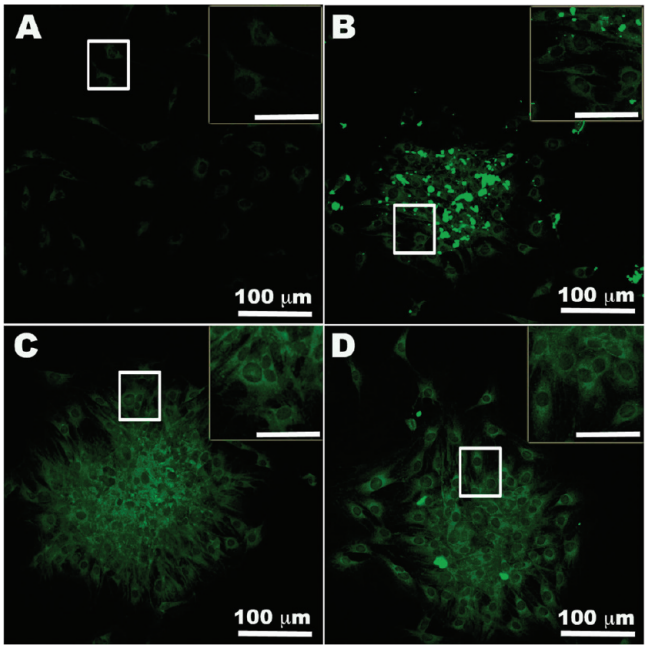


Figure 6. Cellular internalization of siRNA (F) in MC3T3-E1.4 cells after 24 h. (A) siRNA(F) only, (B) siRNA(F) complexed with N-TER, (C) siRNA(F) complexed with N-SS, and (D) siRNA(F) complexed with QN-SS. Increased cellular internalization was observed for cells incubated with star polymer (C and D) compared to N-TER (B). The control group (A) showed weak fluorescence intensity, which could be due to poor cellular internalization of "naked" siRNA (F). Representative images from lower magnification (white box) are shown as higher magnification insets; scale bar = 50 μm.

in the presence of GSH, the most abundant intracellular thiol (0.2−10.0 mM).[72]

Star polymer with a M_w/M_n of 1.33−1.39 and a small size in aqueous media (diameter ~20 nm) were prepared via an "*arm-first*" method in the presence of SS, DMAEMA (or QDMAEMA), and PEGMA macromonomers under conditions that avoided star−star coupling reactions.[73] The residual copper amount after purification was below 24 ppm, which amount is below toxic to cells.[74] Globular star polymers with a covalently stabilized core and segmented core/shell structures are more stable than structures formed from self-assembled templates of organic surfactants and amphiphilic block copolymers, such as micelles and

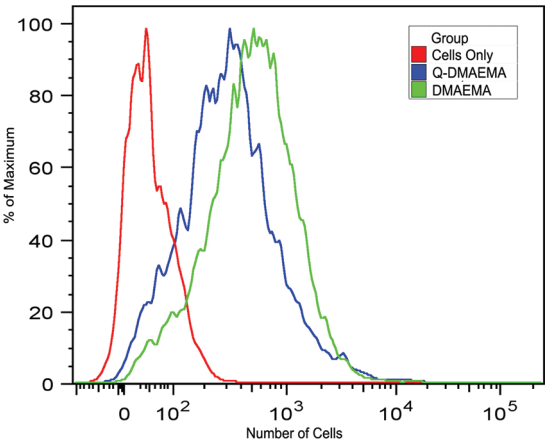


Figure 7. Flow cytometry analysis of cellular internalization of siRNA complexed star polymer at 24 h. N-SS (green color), QN-SS (blue color), and cells only (red color).

vesicles.[54] Star polymers prepared with SS in the core degraded within 48 h in the presence of GSH (100 mM) into individual polymeric chains (Figure 1),[60,65] whereas the nondegradable EGDMA star polymers remained intact even after 7 days under the same conditions. Under a physiological GSH concentration (10 mM), N-SS and QN-SS were successfully degraded after 4 days, while 0-SS was degraded after 6 days due to a different core composition. N-SS and QN-SS compared to 0-SS have more loose cores due to the incorporation of cationic monomers. This may affect the rate of degradation under the same conditions. The ability to make different core compactness has advantages of designing materials with controlled degradability, release properties, and biocompatibility.

Live/dead (Figure 2) and MTS (Figure 3) assays confirmed that the star polymers were nontoxic and biocompatible at 100, 200, 400, and 800 μg/mL concentrations compared to PEI, which is lethal (LC_{50}) at a concentration of 20 μg/mL.[75] It is noteworthy that neither the cationic surface charge nor the molecular weight of the star polymers affected the cell viability (Figure 3) and cell proliferation (Figure 4) compared to PEI,[76] which had significant cytotoxicity at higher concentrations ($P < 0.01$). Due to the structure of prepared star polymers, the materials were biocompatible in comparison to the star polymers with external unprotected PDMAEMA arms for gene delivery systems.[56,69]

Cationic nanoparticles readily form a stable polyplex with negatively charged nucleic acids.[22] Likewise, N-SS and QN-SS form similar-sized stable complexes with siRNAs (Figure 5), as confirmed by the zeta potential values shifted to more negative values after complexation with siRNAs. Based on the zeta potential analysis, both N-SS and QN-SS showed positive values due to the presence of cationic monomers, whereas 0-SS showed a negative value. The nitrogen/phosphate ratio (N/P) is important for gene delivery and can affect cytotoxicity and transfection efficiency. The N/P ratio was calculated based on the number of moles of amine groups in the cationic polymers to phosphate groups in siRNA.[77] N-SS contain 22 nitrogen units in one star molecule (22 units of DMAEMA in a star core). N/P = 1.05 solution was prepared with 2 mol of star polymers (44 nitrogens) and 1 mol of siRNA (42 phosphates). Zeta potential and hydrodynamic diameter were measured for various N/P ratios. Hydrodynamic diameter values were not affected by the N/P

ratio; however, zeta potential values varied depending on the N/P ratio.[75] The addition of PEG to cationic polymers improves delivery of nucleic acid complexes and favors stabilization against aggregation under conditions containing salt and serum.[78] Also, incorporation of a PEG layer can reduce the barrier for adhesion and thereby increase cellular uptake of cationic polymers.[79] However, PEG arms can reduce the strength of complexes between cationic cores and siRNA. This may be considered an advantage for more efficient release of siRNA inside cells.

Finally, the cellular internalization of star polymer polyplexes was evident in MC3T3-E1.4 cells after 24 h incubation, as confirmed by confocal microscopy (Figure 6 and Figure S1) and FACS analysis (Figure 7). Our results are in agreement with Xiao et al.[80] and others[81] who reported cellular uptake efficiency of cationic nanoparticles to be higher than neutral or negatively charged polymers. Particle size may play an important role in cellular internalization. Based on the hydrodynamic diameter, nanomaterials of size >50−60 nm should undergo clathrin-dependent endocytosis, but particles of size <25 nm, as prepared in this study, star polymers with PEG arms and with a cationic core, should undergo caveolae-mediated endocytosis[82] in which the pH remains neutral. This suggests that siRNAs delivered using star polymers should be stable and not undergo lysosomal degradation.

5. CONCLUSIONS

Star polymers with PEG arms and cores containing cationic and degradable units were prepared for siRNA delivery by an "*arm-first*" ATRP method. The star polymers degraded under a supraphysiological and a physiological concentration of GSH into individual polymeric chains due to the incorporation of disulfide units into the cross-linked core. The cytotoxicity of the star polymers was examined in the presence of MC3T3-E1.4 cells after 24 h incubation using live/dead staining. MTS assay results suggested that the addition of a higher concentration of star polymers (100−800 μg/mL) neither produced significant cytotoxicity nor inhibited cell proliferation, suggesting the synthesized polymers were biocompatible. Further, polymer composition was calculated based on GC analysis and $M_{w,MALLS}$ results to determine the number of nitrogens in a star polymer. Reduced zeta potential values of star polymers after siRNA complexation were observed due to complexation with the negatively charged siRNA to the cationic core, while hydrodynamic diameter did not change significantly. Successful cellular uptake of siRNA complexed star polymers was confirmed using confocal laser microscope and flow cytometry. The data presented therefore underscores the exciting future opportunities that may be exploited for siRNA delivery with star polymers with PEG arms and cores containing cationic and degradable units.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Confocal microscopy z-stack images. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: km3b@andrew.cmu.edu.

Author Contributions

†Both authors contributed equally to this work.

■ ACKNOWLEDGMENT

The financial support from DMRDP (DoD) Grant W81XWH1120073, the NSF (DMR 09-69301), and WCU (World Class University) program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (R33-10035-0) is acknowledged. We also would like to thank Dr. James Spanswick for helpful discussions.

■ REFERENCES

(1) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998**, *391* (6669), 806–11.
(2) Hamilton, A. J.; Baulcombe, D. C. *Science* **1999**, *286* (5441), 950–52.
(3) Schiffelers, R. M.; Ansari, A.; Xu, J.; Zhou, Q.; Tang, Q.; Storm, G.; Molema, G.; Lu, P. Y.; Scaria, P. V.; Woodle, M. C. *Nucleic Acids Res.* **2004**, *32* (19), e149.
(4) Bitko, V.; Musiyenko, A.; Shulyayeva, O.; Barik, S. *Nat. Med.* **2005**, *11* (1), 50–5.
(5) Poy, M. N.; Eliasson, L.; Krutzfeldt, J.; Kuwajima, S.; Ma, X.; Macdonald, P. E.; Pfeffer, S.; Tuschl, T.; Rajewsky, N.; Rorsman, P.; Stoffel, M. *Nature* **2004**, *432* (7014), 226–30.
(6) Leachman, S. A.; Hickerson, R. P.; Hull, P. R.; Smith, F. J.; Milstone, L. M.; Lane, E. B.; Bale, S. J.; Roop, D. R.; McLean, W. H.; Kaspar, R. L. *J. Dermatol. Sci.* **2008**, *51* (3), 151–7.
(7) Dykxhoorn, D. M.; Lieberman, J. *Annu. Rev. Med.* **2005**, *56*, 401–23.
(8) de Fougerolles, A.; Vornlocher, H.-P.; Maraganore, J.; Lieberman, J. *Nat. Rev. Drug Discovery* **2007**, *6* (6), 443–53.
(9) Tijsterman, M.; Ketting, R. F.; Plasterk, R. H. *Annu. Rev. Genet.* **2002**, *36*, 489–519.
(10) Gilmore, I. R.; Fox, S. P.; Hollins, A. J.; Sohail, M.; Akhtar, S. *J. Drug Target.* **2004**, *12* (6), 315–40.
(11) Akhtar, S.; Benter, I. *Adv. Drug Delivery Rev.* **2007**, *59* (2−3), 164–82.
(12) Whitehead, K. A.; Langer, R.; Anderson, D. G. *Nat. Rev. Drug Discovery* **2009**, *8* (2), 129–38.
(13) Thomas, C. E.; Ehrhardt, A.; Kay, M. A. *Nat. Rev. Genet.* **2003**, *4* (5), 346–58.
(14) Whitehouse, A. *Int. J. Mol. Med.* **2003**, *11* (2), 139–48.
(15) Fichter, K. M.; Zhang, L.; Kiick, K. L.; Reineke, T. M. *Bioconjugate Chem.* **2008**, *19* (1), 76–88.
(16) Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84* (21), 7413–7.
(17) Chen, G.; Kumar, J.; Gregory, A.; Stenzel, M. H. *Chem. Commun. (Cambridge, U.K.)* **2009**, *41*, 6291–6293.
(18) Kim, W. J.; Kim, S. W. *Pharm. Res.* **2009**, *26* (3), 657–66.
(19) Zhang, L.; Nguyen, T. L. U.; Bernard, J.; Davis, T. P.; Barner-Kowollik, C.; Stenzel, M. H. *Biomacromolecules* **2007**, *8* (9), 2890–2901.
(20) Kakizawa, Y.; Furukawa, S.; Kataoka, K. *J. Controlled Release* **2004**, *97* (2), 345–56.
(21) Higuchi, Y.; Kawakami, S.; Hashida, M. *BioDrugs* **2010**, *24* (3), 195–205.
(22) Han, S.; Mahato, R. I.; Sung, Y. K.; Kim, S. W. *Mol. Ther.* **2000**, *2* (4), 302–17.
(23) Fischer, P. M. *Med. Res. Rev.* **2007**, *27* (6), 755–95.
(24) Zabner, J.; Fasbender, A. J.; Moninger, T.; Poellinger, K. A.; Welsh, M. J. *J. Biol. Chem.* **1995**, *270* (32), 18997–9007.
(25) Carr, L. R.; Jiang, S. *Biomaterials* **2010**, *31* (14), 4186–93.
(26) Oh, J. K.; Bencherif, S. A.; Matyjaszewski, K. *Polymer* **2009**, *50* (19), 4407–23.
(27) Braunecker, W. A.; Matyjaszewski, K. *Prog. Polym. Sci.* **2007**, *32* (1), 93–146.
(28) di Lena, F.; Matyjaszewski, K. *Prog. Polym. Sci.* **2010**, *35* (8), 959–1021.

(29) Heredia, K. L.; Nguyen, T. H.; Chang, C.-W.; Bulmus, V.; Davis, T. P.; Maynard, H. D. *Chem. Commun.* **2008**, *28*, 3245–3247.
(30) Convertine, A. J.; Diab, C.; Prieve, M.; Paschal, A.; Hoffman, A. S.; Johnson, P. H.; Stayton, P. S. *Biomacromolecules* **2010**, *11* (11), 2904–2911.
(31) Gunasekaran, K.; Nguyen, T. H.; Maynard, H. D.; Davis, T. P.; Bulmus, V. *Macromol. Rapid Commun.* **2011**, *32* (8), 654–659.
(32) Convertine, A. J.; Benoit, D. S. W.; Duvall, C. L.; Hoffman, A. S.; Stayton, P. S. *J. Controlled Release* **2009**, *133* (3), 221–229.
(33) York, A. W.; Zhang, Y.; Holley, A. C.; Guo, Y.; Huang, F.; McCormick, C. L. *Biomacromolecules* **2009**, *10* (4), 936–943.
(34) Matyjaszewski, K.; Xia, J. *Chem. Rev.* **2001**, *101* (9), 2921–90.
(35) Patten, T. E.; Xia, J.; Abernathy, T.; Matyjaszewski, K. *Science* **1996**, *272* (5263), 866–8.
(36) Magenau, A. J. D.; Strandwitz, N. C.; Gennaro, A.; Matyjaszewski, K. *Science* **2011**, *332* (6025), 81–4.
(37) Wang, J.-S.; Matyjaszewski, K. *J. Am. Chem. Soc.* **1995**, *117* (20), 5614–5615.
(38) Tsarevsky Nicolay, V.; Matyjaszewski, K. *Chem. Rev.* **2007**, *107* (6), 2270–99.
(39) Matyjaszewski, K.; Tsarevsky, N. V. *Nat. Chem.* **2009**, *1* (4), 276–88.
(40) Siegwart, D. J.; Srinivasan, A.; Bencherif, S. A.; Karunanidhi, A.; Oh, J. K.; Vaidya, S.; Jin, R.; Hollinger, J. O.; Matyjaszewski, K. *Biomacromolecules* **2009**, *10* (8), 2300–9.
(41) Bencherif, S. A.; Siegwart, D. J.; Srinivasan, A.; Horkay, F.; Hollinger, J. O.; Washburn, N. R.; Matyjaszewski, K. *Biomaterials* **2009**, *30* (29), 5270–8.
(42) Oh, J. K.; Drumright, R.; Siegwart, D. J.; Matyjaszewski, K. *Prog. Polym. Sci.* **2008**, *33* (4), 448–477.
(43) Lutz, J.-F.; Boerner, H. G. *Prog. Polym. Sci.* **2008**, *33* (1), 1–39.
(44) Yang, C.; Li, H.; Goh, S. H.; Li, J. *Biomaterials* **2007**, *28* (21), 3245–54.
(45) Pafiti, K. S.; Mastroyiannopoulos, N. P.; Phylactou, L. A.; Patrickios, C. S. *Biomacromolecules* **2011**, *12* (5), 1468–1479.
(46) Georgiou, T. K.; Vamvakaki, M.; Patrickios, C. S.; Yamasaki, E. N.; Phylactou, L. A. *Biomacromolecules* **2004**, *5* (6), 2221–2229.
(47) Georgiou, T. K.; Vamvakaki, M.; Phylactou, L. A.; Patrickios, C. S. *Biomacromolecules* **2005**, *6* (6), 2990–2997.
(48) Georgiou, T. K.; Phylactou, L. A.; Patrickios, C. S. *Biomacromolecules* **2006**, *7* (12), 3505–3512.
(49) Yamaguchi, N.; Kiick, K. L. *Biomacromolecules* **2005**, *6* (4), 1921–30.
(50) Yang, Y. Y.; Wang, Y.; Powell, R.; Chan, P. *Clin. Exp. Pharmacol. Physiol.* **2006**, *33* (5−6), 557–62.
(51) Gao, H.; Matyjaszewski, K. *Macromolecules* **2007**, *40* (3), 399–401.
(52) Hadjichristidis, N. *J. Polym. Sci., Part A: Polym. Chem.* **1999**, *37* (7), 857–71.
(53) Hadjichristidis, N.; Iatrou, H.; Pitsikalis, M.; Mays, J. *Prog. Polym. Sci.* **2006**, *31* (12), 1068–132.
(54) Gao, H.; Matyjaszewski, K. *Prog. Polym. Sci.* **2009**, *34* (4), 317–50.
(55) Soliman, G. M.; Sharma, R.; Choi, A. O.; Varshney, S. K.; Winnik, F. M.; Kakkar, A. K.; Maysinger, D. *Biomaterials* **2010**, *31* (32), 8382–92.
(56) Dai, F.; Sun, P.; Liu, Y.; Liu, W. *Biomaterials* **2010**, *31* (3), 559–69.
(57) Cho, H. Y.; Gao, H.; Srinivasan, A.; Hong, J.; Bencherif, S. A.; Siegwart, D. J.; Paik, H.-j.; Hollinger, J. O.; Matyjaszewski, K. *Biomacromolecules* **2010**, *11* (9), 2199–203.
(58) Etrych, T.; Strohalm, J.; Kovar, L.; Kabesova, M.; Rihova, B.; Ulbrich, K. *J. Controlled Release* **2009**, *140* (1), 18–26.
(59) Sulistio, A.; Widjaya, A.; Blencowe, A.; Zhang, X.; Qiao, G. *Chem. Commun.* **2011**, *47* (4), 1151–3.
(60) Oh, J. K.; Siegwart, D. J.; Lee, H.-i.; Sherwood, G.; Peteanu, L.; Hollinger, J. O.; Kataoka, K.; Matyjaszewski, K. *J. Am. Chem. Soc.* **2007**, *129* (18), 5939–45.
(61) Oh, J. K.; Siegwart, D. J.; Matyjaszewski, K. *Biomacromolecules* **2007**, *8* (11), 3326–31.
(62) Oh, J. K.; Tang, C.; Gao, H.; Tsarevsky, N. V.; Matyjaszewski, K. *J. Am. Chem. Soc.* **2006**, *128* (16), 5578–84.
(63) Lin, C.-C.; Anseth, K. S. *Pharm. Res.* **2009**, *26* (3), 631–643.
(64) Xia, J. H.; Zhang, X.; Matyjaszewski, K. *Macromolecules* **1999**, *32* (13), 4482–4484.
(65) Gao, H.; Tsarevsky, N. V.; Matyjaszewski, K. *Macromolecules* **2005**, *38* (14), 5995–6004.
(66) Tsarevsky, N. V.; Matyjaszewski, K. *Macromolecules* **2005**, *38* (8), 3087–92.
(67) Jones, R. A.; Poniris, M. H.; Wilson, M. R. *J. Controlled Release* **2004**, *96* (3), 379–91.
(68) van de Wetering, P.; Cherng, J.-Y.; Talsma, H.; Hennink, W. E. *J. Controlled Release* **1997**, *49* (1), 59–69.
(69) Alhoranta, A. M.; Lehtinen, J. K.; Urtti, A. O.; Butcher, S. J.; Aseyev, V. O.; Tenhu, H. J. *Biomacromolecules* **2011**ASAP.
(70) Kakizawa, Y.; Kataoka, K. *Adv. Drug Delivery Rev.* **2002**, *54* (2), 203–22.
(71) Liu, J.; Liu, H; Jila, Z; Bulmus, V; Davis, T. P. *Chem. Commun.* **2008**, *48*, 6582–4.
(72) Anderson, M. E. *Chem. Biol. Interact.* **1998**, *111−112*, 1–14.
(73) Gao, H.; Ohno, S.; Matyjaszewski, K. *J. Am. Chem. Soc.* **2006**, *128* (47), 15111–3.
(74) Grillo, C. A.; Reigosa, M. A.; de Mele, M. A. *Contraception* **2010**, *81* (4), 343–9.
(75) Wang, J.; Gao, S. J.; Zhang, P. C.; Wang, S.; Mao, H. Q.; Leong, K. W. *Gene Ther.* **2004**, *11* (12), 1001–10.
(76) Wetering, P.; Moret, E. E.; Schuurmans-Nieuwenbroek, N. M.; Steenbergen, M. J.; Hennink, W. E. *Bioconjugate Chem.* **1999**, *10* (4), 589–97.
(77) Zhao, Q. Q.; Chen, J. L.; Lv, T. F.; He, C. X.; Tang, G. P.; Liang, W. Q.; Tabata, Y.; Gao, J. Q. *Biol. Pharm. Bull.* **2009**, *32* (4), 706–10.
(78) Verbaan, F. J.; Oussoren, C.; van Dam, I. M.; Takakura, Y.; Hashida, M.; Crommelin, D. J.; Hennink, W. E.; Storm, G. *Int. J. Pharm.* **2001**, *214* (1−2), 99–101.
(79) Dan, N. *Biochim. Biophys. Acta, Biomembr.* **2002**, *1564* (2), 343–348.
(80) Xiao, K.; Li, Y.; Luo, J.; Lee, J. S.; Xiao, W.; Gonik, A. M.; Agarwal, R. G.; Lam, K. S. *Biomaterials* **2011**, *32* (13), 3435–46.
(81) Champion, J. A.; Mitragotri, S. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103* (13), 4930–4.
(82) Conner, S. D.; Schmid, S. L. *Nature* **2003**, *422* (6927), 37–44.