Article

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Cationic Nanostructured Polymers for siRNA Delivery in Murine Calvarial Pre-Osteoblasts

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The endogenous RNA interference (RNAi) pathway enables control of pathologies caused by the dysregulation of proteins. Several biological molecules are active in RNAi including short interfering ribonucleic acid (siRNA). The effective utilization of siRNA as a therapeutic agent has been marked with distinct challenges, namely in intracellular delivery and achieving a sufficient dosage to affect protein expression. A delivery strategy we have developed to improve safety and efficacy of siRNA includes complexing siRNA with nanostructured polymers delivery systems (NSPs). These NSPs are synthesized via atom transfer radical polymerization (ATRP) and combine several important advances in polymer architecture for siRNA delivery. This includes shielding the cationic charge of the NSP with a poly(ethylene glycol) (PEG) shell to promote cell viability in MC3T3-E1.4 pre-osteoblasts, and minimize the inflammatory response in a C57BL/6 mouse model. In our gene knockdown experiments targeting glyceraldehyde 3-phosphate dehydrogenase *Gapdh* expression, star polymer and nanogel polyplexes suppressed *Gapdh* mRNA to levels comparable to cells treated with Lipofectamine® RNAiMAX lipoplexes.

KEYWORDS: *Nanostructured Polymer, Star Polymer, Nanogel, siRNA Delivery, Gapdh Knockdown, ATRP.*

INTRODUCTION

The discovery of RNA interference (RNAi) has revolutionized the medical field by providing a new method of control in biological systems. In doing so, the RNAi pathway provides a new class of therapeutic modalities to treat diseases whose etiologies implicate dysregulated proteins.[1–3] Short interfering ribonucleic acids (siRNAs) have been studied extensively since identification by Mello and Andrew Fire.[4] Upon cell internalization, the siRNA becomes part of an RNA-induced silencing complex (RISC), a multiple turnover enzyme that uses the guide (antisense) strand to selectively target and degrade mRNA with complementary sequences.[5] The consequence of this biological event is the reduced expression of the translated protein.

The siRNA strategy is therapeutically compelling, but there are challenges with siRNA-based therapeutics that include siRNA design, toxicity, specificity, off target effects, as well as delivery. The poor drug-like properties of siRNA including its polyanionic backbone results in electrostatic repulsion with the cell membrane, thus decreasing internalization and subsequent transfection efficiency.[6] Furthermore, exogenous and intracellular nucleases may degrade the siRNA's prior to RISC binding. Consequently, unbound siRNAs require a delivery vehicle.

Current technologies used to deliver siRNA's are classified broadly as viral and non-viral vectors.[7] Viral vectors include enveloped and non-enveloped. The enveloped vectors for RNA are retroviruses and lentiviruses, and both may be oncogenic. The non-enveloped vectors are adeno-associated viruses (AAV) and adenoviruses. The main issue with AAVs is a limited capacity for complexation, whereas the capsids of adenoviruses may cause an inflammatory response.[8] Moreover, viral vectors may lead to host resistance when subjected to repeated doses.[8] Non-viral vectors are include cationic lipids,[9] polymers,[10–13] dendrimers,[14–16] carbon nanotubes,[17] as well as cationic peptides[18] and proteins.[19] While cationic lipids facilitate cellular uptake and protection of siRNA's, they may

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be toxic.[20–22] Furthermore, lipid based delivery systems have a tendency to aggregate under serum conditions and can interact with serum proteins.[23–25] Dendrimers are biocompatible,[26] but are associated with complex synthesis and high manufacturing costs. Consequently, there is compelling interest on delivery vehicles that are biocompatible, customizable, and easily synthesized. To address these challenges, nanostructured polymers (NSPs) such as star polymers[27,28] and nanogels[29] are currently being evaluated for drug and gene delivery.

NSPs comprised of poly(ethylene glycol) (PEG), quaternized dimethylaminoethyl methacrylate (qDMAEMA), and a disulfide crosslinker were synthesized via atom transfer radical polymerization (ATRP).[30,31] and tuned for biocompatibility and nucleic acid complexation. We previously characterized the NSP star polymers[32,33] and nanogels,[34] established its biodegradability in the presence of glutathione, and determined their optimal siRNA delivery ratios to achieve maximum knockdown of a reporter gene in a *Drosophila* S2 cell line.[35,36]

In this study, we report on the *in vitro* biocompatibility, complexation, and gene knockdown of these NSPs in a mammalian cell line, murine calvarial preosteoblast-like cells embryonic day 1 subclone 4 (MC3T3-E1.4). The MC3T3-E1.4 cell line has been extensively studied as an osteoprogenitor model,[37] and undergoes osteogenic differentiation and mineralization in a process similar to *in vivo* intramembranous osteogenesis.[38] Therefore, it can be used as a relevant model for studying osteochondral pathologies. Additionally, we report an *in vivo* biocompatibility assessment in a C57BL/6J mouse model.

MATERIALS AND METHODS

NSP Synthesis

Star polymers were prepared using ATRP with the arm-macromonomer approach. Wherein, poly(ethylene glycol) methacrylate ($M_n = 2{,}000$) was copolymerized with DMAEMA and a disulfide dimethacrylate crosslinker with the initiator ethyl 2-bromoisobutyrate and a copper bromide 1,1,4,7,10,10-Hexamethyltriethylenetetramine (HMTETA) catalyst system. The star polymers were purified using dialysis and characterized using gel permeation chromatography. Additional details for the star polymer synthesis and characterization can be found in the following references.[33,35]

Cationic nanogels were prepared by activators generated by electron transfer atom transfer radical polymerization in inverse miniemulsion by copolymerizing quaternized DMAEMA, oligo(ethylene oxide) methacrylate ($M_n = 300$), and a water soluble disulfide methacrylate crosslinker with a poly(ethylene glycol 2-bromoisobutyrate initiator and a copper bromide tris[2-(dimethylamino)-ethyl]amine; catalyst system dissolved in water. The inverse miniemulsion was prepared by ultra-sonication of the aqueous phase in a cyclohexane Span80 solution. After the reaction mixture was degassed an ascorbic acid solution was injected to generate the active catalyst. The cationic nanogels were purified using dialysis and were characterized using dynamic light scattering and zeta potential analysis. Additional details for the star polymer synthesis and characterization can be found in the following Ref. [36]

Cell Culture

MC3T3-E1.4 murine calvarial pre-osteoblasts were purchased from American Type Culture Collection (Manassas, VA), and cultured in alpha-minimum essential medium (α-MEM) and supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cell culture flasks and tissue culture plates used were obtained from Corning. Cell passaging was accomplished with 0.25% Trypsin-ethylenediaminetetraacetic acid (EDTA) (ATCC, Manassas, VA).

Biocompatibility Assessment of NSPs

In Vitro Biocompatibility

A CellTiter 96® AQueous One Solution Assay (Promega, Fitchburg, Wisconsin) was utilized to measure formazan production. Cells were seeded at a density of 20,000/well in a 48 well plate (Sigma-Aldrich, St. Louis, MO) and cultured for 48 hours before being exposed to polymer. For the experimental groups, media containing specific concentrations of NSPs were added. After culturing for an additional 48 hours, MTS reagent was added to each well and incubated at 37 °C and 5% CO_2 for 20–30 minutes. The absorbance was measured at 492 nm using a Tecan Spectra Fluor (Tecan, San Jose, CA). The percentage of cell viability was normalized to cells alone, which was set to 100% cell viability. Branched 25 kDA polyethyleneimine (PEI) (Sigma-Aldrich, St. Louis, MO) at 100 μg/mL was used as a negative control (a decrease in cell viability expected). A cells alone group (no treatment) served as the positive control, in which cell viability was anticipated.

In Vivo Biocompatibility

Synthes xenogeneic collagen matrix (XCM™) scaffolds were loaded with 300 μg NSP each and implanted in the thigh muscle of C57BL/6J mice (8 weeks old). After 4 weeks, muscle implants were recovered and prepared for histology. Histological preparation included dehydration in ethanol, followed by infiltration and embedding in paraffin, and five μm-thick sections were prepared and stained with hematoxylin and eosin (H&E). Slides were imaged at 5.0× magnification using a Zeiss Axiophot (Carl Zeiss Microscopy, Thornwood, NY), and micrographs taken with a Zeiss AxioCam HRc (Carl Zeiss Microscopy, Thornwood, NY). A representative specimen from each cohort is presented.

Complexation with siRNA

NSPs were complexed with negative control siRNA (Invitrogen, Carlsbad, CA) for 1 hour at 4 °C in nuclease free water (Invitrogen, Carlsbad, CA). Samples of polyplex solutions at specific NSP:siRNA weight ratios (w/w) were loaded into a 2% agarose gel in TBE buffer and run at 100 V for 30 minutes. The gel was stained with ethidium bromide at 0.5 μg/μL for 1 hour and imaged under UV transillumination (365 nm).

Gene Knockdown

Star Polymer-Gapdh siRNA Polyplex Knockdown

MC3T3-E1.4 cells were plated at a density of 50,000/well in a 24-well plate one day prior to siRNA transfection. On the day of transfection, star polymers were complexed with 1 μg Silencer® *Gapdh* siRNA (Invitrogen, Carlsbad, CA) at NSP:siRNA weight ratios of 1000:1, 200:1 and 5:1 for 60 minutes at room temperature. Serum free Opti-MEM® medium was added to reach a final volume of 500 μL for each well. The polyplexes were incubated in cell culture for 6 hours and an additional 500 μL Opt-MEM® with 20% FBS was supplemented to each well to reach the final concentration of 10% FBS. After 48 hours, cells were lysed and subjected to the CellsDirect™ One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). A commercial liposomal transfection reagent, Lipofectamine® RNAiMAX (Life Technologies, Carlsbad, CA) was used as a positive control for transfection efficiency.

Nanogel-Gapdh siRNA Polyplex Knockdown

MC3T3-E1.4 cells were plated at the density of 50,000/well in a 24-well plate one day prior to siRNA transfection. On the day of transfection, nanogel polymers were complexed with 1 μg *Gapdh* siRNA at NSP:siRNA weight ratios of 20:1, 5:1 and 1:1 for 60 minutes at room temperature. Serum free Opti-MEM® medium was added to reach a final volume of 500 μL for each well. The polyplexes were incubated in cell culture for 6 hours and an additional 500 μL Opt-MEM® with 20% FBS was supplemented to each well to reach the final concentration of 10% FBS. After 48 hours, cells were lysed and subjected to the CellsDirect™ One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). A commercial liposomal transfection reagent, Lipofectamine® RNAiMAX (Life Technologies, Carlsbad, CA) was used as a positive control for transfection efficiency.

Real-Time PCR Analysis

CellsDirect™ One-Step qRT-PCR Kit with ROX (Life Technologies, Carlsbad, CA) and an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) were used for the analysis of *Gapdh* mRNA expression. Cell lysis, RNA extraction, and cDNA preparation were carried out according to the CellsDirect™ One-Step qRT-PCR (Applied Biosystems, Foster City, CA) Kit protocol. Each reaction contained SuperScript® III RT/Platinum® *Taq* Mix, 2X Reaction Mix with ROX, and Taqman® Gene Expression Assay containing pre-designed primers, and 4 μL of processed cell lysates. All samples were analyzed for Rn18S expression in parallel in the same run as the internal control. Real-time PCR data were represented as Ct values. The comparative Ct method was used to compare the different RNA samples, and RNA expressions in experimental samples were compared to that of the control. Data were expressed as mean ± standard deviation of the relative gene expression fold change.

Statistics

The MTS and *Gapdh* knockdown experiments were performed in triplicate. Data from these experiments are reported as mean ± standard deviation, and statistically compared by one-way ANOVA. A post hoc Fisher's test for multiple comparison was performed if the ANOVA is significant ($P < 0.05$) using Minitab 16 Statistical Software.

RESULTS

Biocompatibility

In Vitro

The MTS assay quantifies cell viability as a correlation to mitochondrial activity. Absorbance values were normalized to the untreated control. The star polymers and nanogels did not induce any significant effect on mitochondrial activity for MC3T3-E1.4 cells from concentrations of 100 to 800 μg/mL. PEI at 100 μg/mL significantly decreased cell viability by 93.6% (Fig. 1).

In Vivo

The Synthes XCM™ scaffold induced a minimum inflammatory cell response that was within acceptable clinical

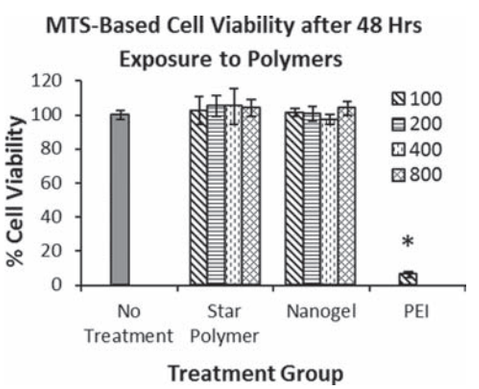

Figure 1. ***In vitro*** **cytotoxicity results for MC3T3-E1.4 cells after 48 hours exposure to star polymers and nanogels at concentrations of 100 to 800 μg/mL. The nanostructured polymers did not significantly reduce mitochondrial enzyme activity. Data are reported as mean ± standard deviation, with samples performed in triplicate. * indicates $P < 0.05$ versus untreated control.**

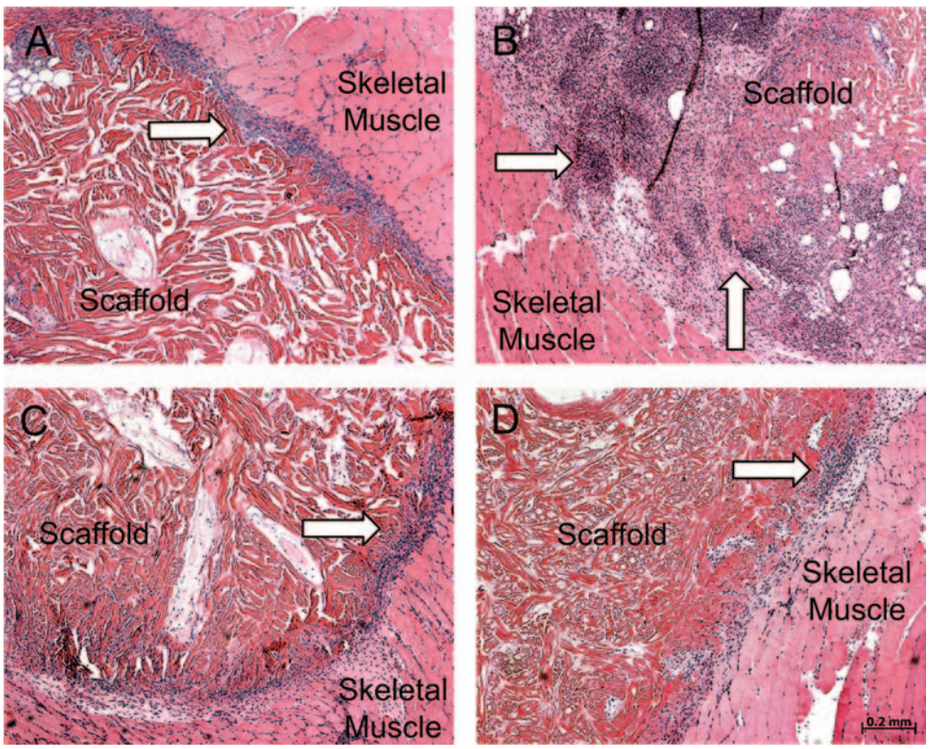

Figure 2. ***In vivo*** **cytotoxicity results for C57BL/6J mice after 4 weeks exposure to NSPs. Mice were implanted with (A) XCM™ collagen scaffold, (B) XCM™ with complete Freund's adjuvant, (C) XCM™ with star polymer, or (D) XCM™ with nanogel. White arrows indicate inflammatory response.**

limits (Fig. 2(A)). The fibrotic responses and levels of inflammatory cell infiltration were slightly increased in mice implanted with scaffolds containing star polymer (Fig. 2(C)) and nanogel (Fig. 2(D)). However, this was deemed to be within normal clinical tolerances. In contrast, the Synthes XCM™ loaded with bacterial extract (complete Freund's adjuvant)[39] (Fig. 2(B)) promoted significant inflammation, an extensive fibrotic response, and necrosis of the surrounding tissue.

Complexation with siRNA

When undergoing electrophoresis, uncomplexed siRNA from each sample migrated towards the cathode due to its anionic charge. When the siRNA in a sample was fully complexed with the NSP, no band was visible on the gel below the wells. The quaternized cationic star polymer achieved full complexation at NSP:siRNA weight ratios of 500:1 w/w (Fig. 3(A)), as indicated by the lack of distinct siRNA bands along those wells. Cationic nanogels were capable of full siRNA complexation at ratios as low as 10:1 w/w (Fig. 3(B)).

Gene Knockdown

Cells treated with star polymer-*Gapdh* siRNA polyplexes knocked down *Gapdh* mRNA expression by $37.9 \pm 16.1\%$ and $37.3 \pm 1.4\%$ when complexed at 200:1 w/w and 1000:1 w/w (NSP:siRNA), respectively (Fig. 4). For the nanogel-*Gapdh* siRNA polyplexes, *Gapdh* mRNA levels were reduced by $43.3 \pm 6.0\%$ and $54.6 \pm 13.2\%$ when using 1:1 w/w and 5:1 w/w (NSP:siRNA), respectively (Fig. 5).

Lipofectamine® RNAiMAX was able to reduce mRNA expression by $70.0 \pm 7.4\%$, demonstrating a higher knockdown efficiency compared to most of groups involving NSPs. However, when analyzed using ANOVA and Fisher's test, no statistical difference was detected when comparing knockdown efficiency of RNAiMAX to the Star 200:1 and Nanogel 5:1 groups.

DISCUSSION

The MTS result (Fig. 1) allowed us to conclude the plasma membrane remained intact, and mitochondrial activity was not disrupted in the presence of the NSPs. While PEI is considered the gold standard among cationic polymers for gene delivery, its cytotoxic effects have been well established and characterized.[40–43] Exposure of cells to PEI led to destabilized membrane integrity followed by mitochondrial induced apoptosis.[42] Therefore, cells treated with PEI decreased mitochondrial enzyme activity significantly. Dimethylaminoethyl methacrylate (DMAEMA) has been investigated as an alternative to PEI, but previous studies indicate cells treated with poly(dimethylaminoethyl methacrylate) (PDMAEMA) also exhibited a decrease in cell viability at concentrations of 200 μg/mL or less when

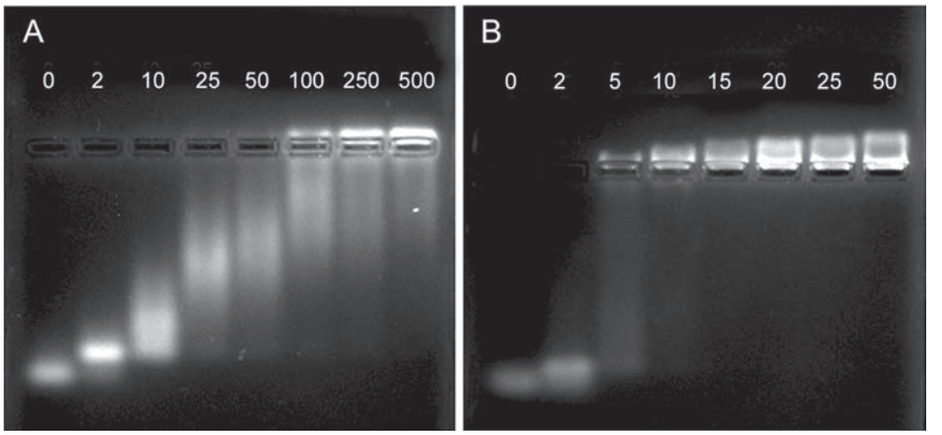

Figure 3. **Gel electrophoresis results for cationic star polymers (A) and cationic nanogels (B). NSP ratios to siRNA are denoted above each well (w/w). Stars fully complex siRNA at weight ratios of 500:1 w/w, whereas nanogels fully complex with siRNA at 10:1 w/w.**

evaluated using an MTT assay.[44–46] The NSPs have surpassed this prior limitation by maintaining cell viability up to 800 μg/mL. The histological images (Fig. 2) also suggest these NSPs are biocompatible *in vivo*. While there is a marginally increased fibrotic response, the NSPs did not illicit necrosis of the surrounding skeletal muscle. Moreover, the inflammatory cell density surrounding the scaffolds containing NSPs was similar to that of the scaffold only group. Consequently, we conclude the PEGylated shells of the star polymers and nanogels likely shielded and mitigated the cytotoxic effects of qDMAEMA.

The complexation data (Fig. 3) suggest nanogels have a higher charge density compared to the star polymer. This phenomenon likely exists due to the nanogel's larger hydrodynamic diameter of approximately 200 nm, compared to 10 nm of the star polymer. The nanogels are also synthesized with shorter PEG chains, which may have enabled increased siRNA complexation.[33, 36]

While there is no statistical difference between the knockdown efficiencies of the Nanogel 5:1 and Star 200:1 groups, lower quantities of nanogels were needed to attain the same level of knockdown as the star polymers. The level of *Gapdh* knockdown in a mammalian cell is consistent with other cationic polymeric systems.[47] The knockdown results also suggest that at optimal ratios, NSPs are capable of inducing a similar level of knockdown as RNAiMAX, while maintaining their biocompatibility. The main limitation of RNAiMAX as a therapeutic

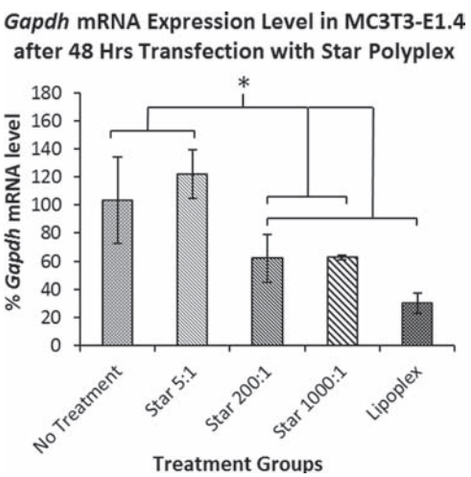

Figure 4. ***Gapdh*** **knockdown levels for MC3T3-E1.4 cells treated with star polymers. Stars polyplexes at weight ratios of 5:1, 200:1, and 1000:1 were used to evaluate** ***Gapdh*** **mRNA knockdown efficiency. The 200:1 ratio induced a knockdown efficiency of 37.9%. Lipoplex (RNAiMAX complexed with siRNA) served as a positive control. Results are reported as mean ± standard deviation, with samples performed in triplicate. * indicates $P < 0.05$ versus untreated control.**

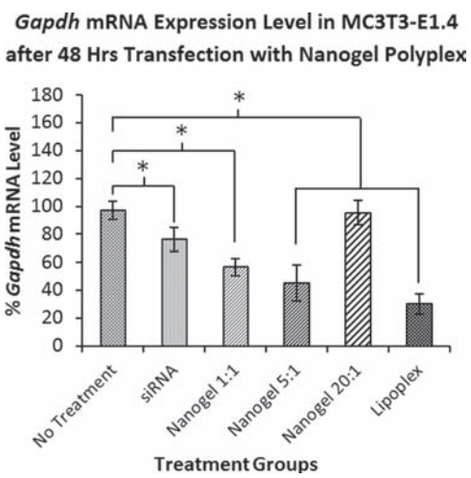

Figure 5. ***Gapdh*** **knockdown levels for MC3T3-E1.4 cells treated with nanogels. Nanogel polyplexes at weight ratios of 1:1, 5:1, and 20:1 were used to evaluate** ***Gapdh*** **mRNA knockdown efficiency. The 5:1 ratio induced a knockdown efficiency of 54.6%. Lipoplex (RNAiMAX complexed with siRNA) served as a positive control. Results are reported as mean ± standard deviation, with samples performed in triplicate. * indicates $P < 0.05$ versus untreated control.**

are cytotoxic effects that reduce cell proliferation[48] and viability.[12] Furthermore, cells treated with RNAiMAX showed a decrease in DNA concentration.

Previous results in the S2 cell line indicate an optimal ratio of 0.2:1 for maximum knockdown.[35,36] This effect may be due to a difference in pH of the two medium. While Schneider's *Drosophila* Media (Sigma-Aldrich, St. Louis, Missouri) normally has a pH of 6.3 to 6.5, α-MEM's pH is typically 7 to 7.4. A higher percentage of the tertiary amine in DMAEMA becomes protonated at lower pH, thereby increasing the cationic charge of the NSP. Thus, transfections conducted in lower pH media require less NSP for complexation and knockdown.

CONCLUSION

Both star polymers and nanogels were biocompatible, complexed and delivered *Gapdh* siRNA, and suppressed *Gapdh* mRNA production to levels comparable to that of Lipofectamine® RNAiMAX. This underscores that the architecture of these NSPs do not compromise biocompatibility while maintaining knockdown efficiency.

Future iterations of the nanostructured polymers may involve incorporating greater amounts of qDMAEMA to improve complexation efficiency while maintaining biocompatibility. The star polymers may be synthesized with a higher charge density by increasing core size as well as cationic content. This would increase the binding affinity of the star polymers to siRNA, thereby reducing the amount of star polymers needed to induce effective knockdown.

Additionally, *Gapdh* mRNA knockdown studies determined the complexation ratios that would result in the highest knockdown efficiency for the MC3T3–E1.4 cell line. Since this cell line is osteoblast-like, it is often exploited for bone studies. The results obtained thus far suggest promise in using the cationic nanostructured polymers to deliver siRNA targeting key osteogenic regulators to abrogate osteoblast pathologies observed in heterotopic ossification and fibrodysplasia ossificans progressiva.

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