Preparation of Cationic Nanogels for Nucleic Acid Delivery


ABSTRACT: Cationic nanogels with site-selected functionality were designed for the delivery of nucleic acid payloads targeting numerous therapeutic applications. Functional cationic nanogels containing quaternized 2-(dimethylamino)ethyl methacrylate and a cross-linker with reducible disulfide moieties (qNG) were prepared by electron transfer atom transfer radical polymerization (ATRP) in an inverse miniemulsion. Polyplex formation between the qNG and nucleic acid exemplified by plasmid DNA (pDNA) and short interfering RNA (siRNA duplexes) were evaluated. The delivery of polyplexes was optimized for the delivery of pDNA and siRNA to the Drosophila Schneider 2 (S2) cell-line. The qNG/nucleic acid (i.e., siRNA and pDNA) polyplexes were found to be highly effective in their capabilities to deliver their respective payloads.

Short interfering RNA (siRNA) and plasmid DNA (pDNA) have emerged as important agents in both basic research and therapeutic strategies.1–4 They affect the biosynthesis of their targeted proteins either through the introduction of specific units, resulting in synthesis of new proteins, in the case of pDNA, or targeting of messenger RNAs (mRNAs) for RNA interference (RNAi), as in the case of siRNA.5,6 The selective delivery of siRNA and pDNA has been a challenge due to their degradation in the presence of nuclease and their anionic charge that hinders their cell permeability.7,8 Solutions include cationic carrier systems, such as cationic lipids or polymers, which generate complexes via electrostatic interactions to form lipoplexes or polyplexes, respectively, and thus enhance the transfection of nucleic acids into cells.9–24 Nevertheless, the design of efficient polyplex-based siRNA and pDNA delivery systems for transfection is itself a challenge that limits realizing the full potential of siRNA and pDNA for therapeutic applications.4,8,25–28

Previously, the preparation of biodegradable cross-linked nanogels (NGs),11 comprising an oligo(ethylene oxide) methacrylate (OEOMA) backbone was demonstrated using electron transfer atom transfer radical polymerization (AGET ATRP) in inverse miniemulsion.29–31 This method was successfully used to prepare protein–polymer hybrid NGs32,33 and NGs for delivery of small molecules34 and carbohydrates.35 However, in order to use these materials for effective nucleic acid delivery, it is necessary to have site-specific incorporation of cationic monomers, into the predominant OEOMA NGs for polyplex formation, but currently this has not been accomplished. ATRP36–39 is a versatile polymerization method that can be applied for the synthesis of diverse and complex polymeric architectures,40–44 including cationic and quaternized nanogels (qNGs). In this communication, we report the synthesis of a biodegradable qNG via the copolymerization of OEOMA and a cationic monomer, quaternized dimethylaminoethyl methacrylate,45,46 by AGET ATRP in inverse miniemulsion. A disulfide-based cross-linker9,30,46–48 was utilized to take advantage of the different concentrations of reducing agent contained in the extracellular (~1 μM) versus intracellular (~10 mM) matrix39,50 and facilitate the biodegradation of the NG after transfection of the nucleic acid cargo. The qNGs were studied for the complexation and delivery of a pDNA that codes for a firefly luciferase protein and siRNA that targets a renilla luciferase mRNA for a dual luciferase reporter assay. The Drosophila Schneider 2 (S2) cell line was used as the model because of its importance and prevalence in basic biological research and challenges in successfully delivering both siRNA and pDNA to these cells.51–69

The biodegradable qNG were prepared in a water-in-oil inverse miniemulsion utilizing AGET ATRP (Scheme 1).71 s1 Cu[0]Br2/tris(2-pyridylmethyl)amine (TPMA) was used as the ATRP catalytic species, poly(ethylene glycol) methyl ether 2–73 bromoisobutyrate (PEGMI2000, Mn = 2000) as a macroinitiator,74 oligo(ethylene oxide)methacrylate (OEOMA300, Mn = 300),75

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and quaternized dimethylaminoethyl methacrylate (Q-DMAEMA-MA, DMAEMA was quaternized with ethyl bromide) as comonomers, dithiopropionyl poly(ethylene glycol) dimethacrylate (DMA) ($M_n=1260$) as a cross-linking agent, and PEGOH2000 as a stabilizer. They were dissolved in 1.40 mL of ultrapure water and added to a 25 mL solution of 0.05% (w/w) of Span-80 in cyclohexane. The reaction mixture was emulsified using ultrasonication to form stable water-in-oil droplets. The mixture was degassed with nitrogen and a degassed solution of ascorbic acid was injected into the emulsion to convert Cu$^{II}$Br$_2$ to Cu$^{I}$Br and initiate the AGET ATRP. The polymerization was carried out for 24 h at 30 °C. The qNGs were purified by precipitation into THF, then washed several times with ultrapure water followed by dialysis (25 k MWCO membrane) against water to remove all unreacted reagents.

The qNGs were characterized by dynamic light scattering (DLS) and zeta potential, revealing a particle size of ca. 275 nm with a PDI of 0.164, $\zeta$ potential of 43.7 mV ±4.1 (Figure 1A). To determine the biodegradability of these qNGs under reducing conditions, a 1 mg/mL solution of qNG in 10 mM glutathione was prepared, and its particle size was monitored using DLS. The resulting degraded particles had a volume distribution of ca. 4 nm in diameter, indicating a successful REDOX mediated degradation of the qNG (Figure 1B).

Scheme 1. Synthesis of Cationic NGs for Nucleotide Delivery of siRNA and pDNA Using AGET ATRP

![Scheme 1](https://example.com/scheme1.png)

Figure 1. Normalized volume distribution of cationic NGs prepared by AGET ATRP in inverse miniemulsion measured using DLS. (A) Volume distribution of purified qNG prepared from PEOM$_{3000}$:OEOMA$_{300}$:Q-DMAEMA:DMA:Cu(II)Br$_2$:Ascorbic Acid: 1/290/20/2/0.5/0.6/0.3, 55 mg PEGOH$_{2000}$ in 5% Span80 in cyclohexane for 24 h at 30 °C. Size = 275 nm, PDI = 0.164, $\zeta$ potential = 43.7 mV ±4.1. (B) Volume distribution of qNG after incubation with 10 mM glutathione for 4 days.

![Figure 1A](https://example.com/figure1a.png)  ![Figure 1B](https://example.com/figure1b.png)

Figure 2. Agarose gel electrophoresis analysis of polyplex formation and disassociation of qNG and pDNA (LacZ-plasmid). Electrophoresis was conducted for 30 min, at 100 V, and the gels were stained with EtBr and imaged with UV-transillumination. (A) Polyplex formation, 500 ng of plasmid was incubated with varying amounts of qNG (R1-R500) for 1 h at 25 °C and then loaded onto a 1% agarose gel electrophoresis. (B) Preformed polyplexes of qNG:pDNA (R1-R200) were incubated with 0.015 μg/μL Heparin sulfate for 30 min and then examined by gel electrophoresis using a 0.5% agarose gel.

![Figure 2A](https://example.com/figure2a.png)  ![Figure 2B](https://example.com/figure2b.png)
The ability of the qNG to complex both pDNA and siRNA was investigated using agarose gel shift assay. This assay was used to determine the weight ratio, R (i.e., NG:siRNA or NG:pDNA), at which qNG totally complexed pDNA and siRNA, respectively. Five hundred nanograms of pDNA was hybridized with qNG for 1 h at 25 °C (in nuclease-free ultrapure water pH = 7) and then loaded onto a 1% agarose gel. Following electrophoresis, the gels were stained with ethidium bromide (EtBr) and imaged. For pDNA, a ratio of R5 (i.e., weightqNG:weightpDNA = 5:1) showed a lack of band migration of the qNG:pDNA polyplex. This indicated a near total complexation of the DNA with the qNG (Figure 2A). A polyplex disassociation study was conducted, by adding heparin sulfate to the precomplexed qNG:pDNA polyplexes, to determine the reversibility of the complexation between qNG and pDNA. At ratios less than R50, the polyplexes could be dissociated with heparin sulfate (0.015 μg/μL) (Figure 2B). This result suggests that, although pDNA could be complexed, it could be released, when a small excess of qNG was used to complex the pDNA. Preliminary characterization of qNG:pDNA polyplexes using DLS and ζ potential analysis was conducted (Figure SI-1, Supporting Information). No polyplex disassociation study was conducted, by adding heparin sulfate (0.015 μg/μL) (Figure 2B).

Polyplexes at (R1500, R300, R30, R15, R3, R0.3 and R0.03) (experimental group). A paired Students t-Test was used to compare the efficacies of the qNG:pDNA polyplexes and FuGENE-HD. The polyplexes prepared at R values of 300, 30, and 15 did not differ significantly from pDNA transfected with FuGene-HD (p > .05).

The qNG’s ability to deliver siRNA was tested using a dual luciferase reporter assay (Dual-Glo luciferase reporter assay, Promega) with S2 cells transfected with both firefly luciferase and Renilla luciferase (RLuc). Three hours after reporter plasmids were transfected, polyplexes of qNG and siRNA against RLuc were formed at R100, R20, R2, R1, R0.2 and R0.02, which were used to determine the optimal siRNA transfection R values. After 24 h, the post siRNA transfection RLuc and FLuc signals were measured, and the RLuc knockdown was quantified and normalized to the FLuc signal and a control well (N = 3). When no transfection reagent (i.e., no FuGene-HD or qNG) was used, the siRNA was inactive (Red bar), suggesting the initial transfection agent used to deliver the plasmid had been washed away. Maximum RLuc reporter signal knockdown was observed at a ratio of R0.2 (Figure 5). A paired Students t-Test was used to compare...
Figure 5. siRNA delivery using a dual-Luciferase reporter assay. Normalized to FLuc, RLuc activity in S2 cells after 24 h treatment with siRNA with no transfection reagent, i.e., siRNA with no FuGENE or qNG (negative control), 9 pmol of duplex siRNA with FuGene-HD (positive control), or a weight ratio of qNG:siRNA at R100, R20, R2, R0.2 and R0.02 (experimental group).

qNG:siRNA polyplexes efficiency compared to FuGENE-HD.

The efficacy of the polyplexes prepared at R values of 2, 1, and 0.2 were found not to differ significantly from the efficacy of siRNA transfection with FuGene-HD (positive control), or a weight ratio of qNG:siRNA at R100, R20, R2, R0.2 and R0.02 (experimental group).

**CONCLUSIONS**

We have demonstrated that well-defined qNGs (275 nm, PDI 0.164) can be prepared using AGET ATRP in inverse miniemulsion. The disulfide cross-linker conferred biodegradability to the qNG, causing it to undergo a REDOX-mediated degradation with glutathione, a model biological reducing agent. Moreover, the qNGs complexed pDNA and siRNA at relatively low weight ratios of qNG to DNA (5:1) and qNG to siRNA (15:1), as determined by agarose gel electrophoresis. Further, the NGs provided a robust delivery system for pDNA (~5 kb) as well as siRNA. On the basis of the electrophoresis dissociation experiments, we hypothesize that a balance between tight binding of nucleic acids to the qNG and ability to dissociate must be achieved for effective release and delivery of complexed material. In order to characterize the ability of different ratios of qNG to transfect siRNA, a dual-luciferase reporter assay was utilized to rapidly and accurately screen knockdown efficiency. A maximum reporter knockdown was obtained at R0.2, with efficacy suggesting more effective transfection than siRNA-FuGene-HD. For pDNA transfection, the maximum firefly luciferase reporter signal was observed at R30. These results confirm that qNGs are a promising platform for pDNA and siRNA delivery and future studies will include clinically relevant mammalian cells treated with the polyplexes.

**ASSOCIATED CONTENT**

See Supporting Information (SI) for detailed NG synthesis, siRNA and pDNA transfection experiments, materials, methods, and additional studies of qNG:pDNA polyplex size and zeta potential characterizations. The qNG’s effect on MC3T3 cell viability can also be found in the SI. This information is available free of charge via the Internet at http://pubs.acs.org/.

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**Author Contributions**

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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