Published in final edited form as:

Biomaterials. 2012 October ; 33(28): 6868–6876. doi:10.1016/j.biomaterials.2012.06.020.

Multifunctional triblock copolymers for intracellular messenger RNA delivery

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Abstract

Messenger RNA (mRNA) is a promising alternative to plasmid DNA (pDNA) for gene vaccination applications, but safe and effective delivery systems are rare. Reversible addition-fragmentation chain transfer (RAFT) polymerization was employed to synthesize a series of triblock copolymers designed to enhance the intracellular delivery of mRNA. These materials are composed of a cationic dimethylaminoethyl methacrylate (DMAEMA) segment to mediate mRNA condensation, a hydrophilic poly(ethylene glycol) methyl ether methacrylate (PEGMA) segment to enhance stability and biocompatibility, and a pH-responsive endosomolytic copolymer of diethylaminoethyl methacrylate (DEAEMA) and butyl methacrylate (BMA) designed to facilitate cytosolic entry. The blocking order and PEGMA segment length were systematically varied to investigate the effect of different polymer architectures on mRNA delivery efficacy. These polymers were monodisperse, exhibited pH-dependent hemolytic activity, and condensed mRNA into 86–216 nm particles. mRNA polyplexes formed from polymers with the PEGMA segment in the center of the polymer chain displayed the greatest stability to heparin displacement and were associated with the highest transfection efficiencies in two immune cell lines, RAW 264.7 macrophages (77%) and DC2.4 dendritic cells (50%). Transfected DC2.4 cells were shown to be capable of subsequently activating antigen-specific T cells, demonstrating the potential of these multifunctional triblock copolymers for mRNA-based vaccination strategies.

Keywords

mRNA; gene therapy; copolymer; micelle; immune response

1. Introduction

Messenger RNA (mRNA) is increasingly being recognized as a promising alternative to plasmid DNA (pDNA) in gene vaccination strategies and has been utilized for the *ex vivo* transfection of dendritic cells for adoptive transfer [1–3] as well as for direct injection [4–6]. A significant advantage of mRNA therapeutics is that they do not require nuclear entry activity for activity, a property that may generate higher and faster levels of gene expression compared to pDNA [7,8]. This is particularly significant for delivery to slow- or non-dividing mammalian cells, whose relative intransigence to pDNA transfection has been attributed to the nuclear entry barrier [9–11]. The clinical application of mRNA-based

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therapies also provides a safety advantage over pDNA-based approaches since mRNA does not integrate into the host genome, allowing for transient gene expression without the risk of insertional mutagenesis.

To improve the effectiveness of mRNA therapeutics, efficient and safe carrier systems are needed to overcome the numerous intra- and extracellular challenges involved in mRNA delivery, including protection from nuclease degradation, escape from endosomal/lysosomal trafficking, and cytoplasmic entry and release [12]. However, few research efforts have been reported to date regarding the development of mRNA-specific delivery vehicles [7,8]. Most of the existing studies have focused on the use of cationic lipids and liposomes such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and Lipofectamine [13,14]. While polymeric vectors have been extensively evaluated as alternatives to liposomal-based systems for the delivery of other nucleic acid therapeutics such as pDNA and small interfering RNA (siRNA), polymer-based mRNA delivery remains relatively unexplored. Previous reports have observed that commonly used cationic homopolymers such as poly(ethyleneimine) (PEI) [14–17], poly(L-lysine) (PLL) [15,16], and poly(dimethylaminoethyl methacrylate) (pDMAEMA) [18] are typically less effective for mRNA delivery, potentially due to differences in mRNA-polycation binding compared to pDNA and siRNA [14,18–20], although high transfection efficiencies have been reported with PEI in certain studies [18,20]. Improvements in mRNA delivery have been achieved with modified polymer systems, including peptide-conjugated low molecular weight PEI [15], reducible histidine-PLL copolymers [17], copolymers of DMAEMA and oligo(ethylene glycol) methyl ether methacrylate [18], ternary complexes of PEI and PEI-poly(ethylene glycol) copolymers [20], ternary complexes of DOTAP and reducible linear poly(amido amines) [19], and lipid-enveloped poly(β -amino ester) nanoparticles [21].

Block copolymer systems allow for the modular incorporation of multiple functionalities into a single polymer structure and have been widely explored for gene delivery applications. Successful polymer architectures have included (A) cationic segments for condensing nucleic acids, (B) hydrophilic segments for enhancing stability and biocompatibility via steric shielding, and (C) pH-responsive segments capable of mediating escape from acidic endosomal/lysosomal compartments [22–24]. Various ABC-type triblock copolymer systems have been previously reported in the context of pDNA [25–31] and siRNA [32–34] transfection, but not for mRNA delivery.

Controlled living radical polymerization methodologies allow for the synthesis of previously unattainable polymer systems with predetermined architectures, well-defined functional segments, and low material heterogeneity, and have been successfully used for the construction of polymeric gene delivery vectors [35]. Previously, we described the reversible addition-fragmentation chain transfer (RAFT) synthesis of micellar diblock copolymers for siRNA and pDNA delivery incorporating a cationic DMAEMA block and a pH-triggered endosomal releasing block composed of propylacrylic acid, butyl methacrylate (BMA) and DMAEMA [36], or diethylaminoethyl methacrylate (DEAEMA) and BMA [37], respectively. Here, we describe a series of ABC-type triblock copolymers for mRNA delivery that are composed of a DMAEMA segment for cation-mediated mRNA binding, a PEGMA segment to impart stabilization via steric shielding, and a copolymer of DEAEMA and BMA (DEAEMA-*co*-BMA) to confer endosomal escape capabilities via a pH-induced transition to a membrane-destabilizing conformation [37]. This polymer series was designed to systematically vary block order and PEGMA block size in order to elucidate the effects of different block copolymer architectures on polyplex characteristics and *in vitro* biological activity.

2. Materials and Methods

2.1 Materials

Chemicals were supplied by Sigma-Aldrich unless otherwise specified. DMAEMA, DEAEMA, and BMA were distilled prior to use. PEGMA (average $M_n = 300$ g/mol) was passed through a basic alumina column prior to use. 2,2-azobisisobutyronitrile (AIBN) was recrystallized from methanol. 4-cyanopentanoic acid dithiobenzoate (CTP) and 4,4-azobis(4-cyanopentanoic acid) (V-501) were used as received.

2.2 RAFT-mediated polymer synthesis

2.2.1 Synthesis of pDMAEMA macro chain transfer agent (macroCTA)—The RAFT polymerization of DMAEMA (6 g, 38.2 mmol) was conducted in dioxane (9 g, 102 mmol) at 60 °C (40 wt% monomer to solvent) under a nitrogen atmosphere for 18 h using CTP (142 mg, 0.509 mmol) and V-501 (36 mg, 0.127 mmol) as the chain transfer agent (CTA) and radical initiator, respectively. The initial monomer to CTA ratio ($[M]_0/[CTA]_0$) and the initial CTA to initiator ratio ($[CTA]_0/[I]_0$) were 75:1 and 4:1, respectively. The resultant macroCTA was isolated by precipitation into pentane (x5) and dried overnight *in vacuo*.

2.2.2 Synthesis of pPEGMA macroCTA—The RAFT polymerization of PEGMA (2 g, 6.67 mmol) was conducted in dioxane (18 g, 204 mmol) at 60 °C (10 wt% monomer to solvent) under a nitrogen atmosphere for 18 h using CTP (29 mg, 0.103 mmol) and AIBN (1.7 mg, 0.0103 mmol), with $[M]_0/[CTA]_0$ and $[CTA]_0/[I]_0$ ratios of 65:1 and 10:1, respectively. The resultant macroCTA was isolated by precipitation and dried.

2.2.3 Synthesis of diblock copolymer DE—DEAEMA (224 mg, 1.21 mmol) and BMA (74 mg, 0.518 mmol) monomers (30% mole fraction BMA) were added to pDMAEMA macroCTA (200 mg, 0.0173 mmol) dissolved in dioxane (746 mg, 8.47 mmol) (40 wt% monomer and macroCTA to solvent) at a $[M]_0/[CTA]_0$ ratio of 100:1. The reaction was conducted at 60 °C under a nitrogen atmosphere for 18 h using V-501 (1.2 mg, 4.32 μ mol) with $[CTA]_0/[I]_0$ ratio 4:1, and the final polymer was isolated by precipitation and dried.

2.2.4 Synthesis of triblock copolymers DPE1, DPE2, DPE3—PEGMA monomer was added to pDMAEMA macroCTA dissolved in dioxane (10 wt% monomer and macroCTA to solvent) at $[M]_0/[CTA]_0$ ratios of 40:1 (DPE1), 100:1 (DPE2), or 150:1 (DPE3). Reactions were conducted at 60 °C under a nitrogen atmosphere using AIBN with $[CTA]_0/[I]_0$ ratio 10:1, and the resultant macroCTAs were isolated by precipitation and dried. These macroCTAs were used in the block copolymerization of DEAEMA and BMA at $[M]_0/[CTA]_0$ ratios of 100:1 (DPE1, DPE2) or 175:1 (DPE3), with all other reaction conditions and purification procedures the same as previously described for polymer DE.

2.2.5 Synthesis of triblock copolymers PDE, PED—Polymer PDE was synthesized in a two-step procedure: (1) block copolymerization of DMAEMA from a pPEGMA macroCTA with $[M]_0/[CTA]_0$ ratio 75:1, followed by (2) block copolymerization of DEAEMA and BMA from the resultant diblock macroCTA with $[M]_0/[CTA]_0$ ratio 100:1. Polymer PED was synthesized by: (1) block copolymerization of DEAEMA and BMA from a pPEGMA macroCTA with $[M]_0/[CTA]_0$ ratio 100:1, followed by (2) block copolymerization DMAEMA from the resultant diblock macroCTA with $[M]_0/[CTA]_0$ ratio 75:1. All other conditions and procedures for the polymerizations of DMAEMA or DEAEMA-*co*-BMA were the same as previously described.

2.2.6 Chromatographic purification and preparation of polymer stock

solutions—Dried polymers were dissolved into ethanol followed by addition into Dulbecco's phosphate-buffered saline (DPBS) (10 vol% ethanol, 8–12 mg/mL polymer), purified by chromatographic separation via a PD-10 desalting column (GE Healthcare), and lyophilized. Polymer stock solutions were prepared by redissolving lyophilized polymers into ethanol followed by addition into DPBS (2 vol% ethanol, 2 mg/mL polymer).

2.3 Nuclear magnetic resonance (NMR) spectroscopy

The ^1H -NMR spectra of all dried macroCTA intermediates and final lyophilized polymers were acquired using a Bruker AV 500 at 10–20 mg/mL polymer in CDCl_3 . The monomer compositions of the DEAEMA-*co*-BMA block were determined by the integration of the peaks described in Fig. 2.

2.4 Gel permeation chromatography (GPC)

Molecular weights and polydispersity indices were determined using Tosoh SEC TSK-GEL R-3000 and R-4000 columns connected in series to an Agilent 1200 series liquid chromatography system, Wyatt miniDAWN TREOS triple-angle MALS light scattering detector, and Optilab-rEX refractive index detector. HPLC-grade DMF containing 0.1 wt% LiBr at 60 °C was used as the mobile phase at a flow rate of 1 mL/min. Polymer molecular weights were determined using dn/dc values calculated separately for each macroCTA and polymer.

2.5 Hemolysis assay

A red blood cell hemolysis assay was performed as previously described [38]. Briefly, polymer solutions were incubated with red blood cells in 100 mM sodium phosphate buffer at pH values of 7.4, 7.0, 6.6, 6.2, or 5.8. In order to normalize hemolytic activity to the total amount of endosomolytic units available per polymer, the concentration of each polymer solution was calculated such that the final mass concentration of the DEAEMA-*co*-BMA block was 20 $\mu\text{g/mL}$. Triton X-100 (1 vol% in water) was used as a positive control. After 1 h incubation at 37 °C, the amount of released hemoglobin was measured via absorbance at 492 nm.

2.6 Plasmids and in vitro transcription of mRNA

Plasmid DNA was prepared using the EndoFree Plasmid Mega Kit (Qiagen). Plasmid pGEM4Z-GFP-A64 (a gift from E. Gilboa, University of Miami) encoding eGFP with a synthetic poly-A tail has been previously described [39]. The GFP coding fragment was excised with XbaI and NotI to produce pGEM4Z-A64. The ovalbumin cDNA EcoRI fragment from plasmid pAc-neo-OVA (a gift from M.J. Bevan, University of Washington) was subcloned into the pVAX1 vector (Invitrogen) and amplified by PCR. The PCR primers used added XbaI and NotI sites to the 5' and 3' ends of the cDNA: 5'-GCTCTAGAATGGGCTCCATCGGTGCAGC-3' (forward) and 5'-GCGCGGCCGCTTAAGGGGAAACACATCTGCC-3' (reverse). The product was ligated into pGEM4Z-A64 to produce pGEM4Z-OVA-A64. pGEM4Z-GFP-A64 and pGEM4Z-OVA-A64 were linearized by SpeI and used as templates for *in vitro* transcription using a mMESSAGE mMACHINE T7 Kit (Ambion). The resultant mRNA was purified using a RNeasy MinElute Cleanup Kit (Qiagen).

2.7 Formation of mRNA polyplexes and lipopolyplexes

Polyplexes were formed by combining equal volumes of mRNA (0.1 $\mu\text{g}/\mu\text{L}$ in nuclease-free water) and polymer solutions for 30 min at room temperature. The theoretical charge ratios (+/−) of the polyplexes were calculated using the mass concentration of the cationic

DMAEMA block, assumed to be 50% protonated at pH 7.4 [40], and the negatively-charged mRNA. All polyplexes in this study were formed at a theoretical charge ratio of 4:1 unless otherwise specified. Lipoplexes were formed by combining mRNA and Lipofectamine 2000 (Invitrogen) at a 3:1 v/w Lipofectamine:mRNA ratio in serum and antibiotic-free media in accordance with the manufacturer's protocol.

2.8 Characterization of polyplex size and zeta potential

Dynamic light scattering (DLS) and zeta potential measurements were conducted using a ZetaSizer Nano ZS (Malvern) at a constant scattering angle of 173° at 25 °C. Measurements for polymer micelles were conducted using 2 mg/mL polymer solutions in DPBS. Polyplexes were diluted to 5 µg/mL mRNA in DPBS or water for size and zeta potential measurements, respectively.

2.9 Heparin competitive displacement assay

Solutions of heparin sodium salt in water were combined with equal volumes of polyplexes containing 0.5 µg mRNA and incubated at room temperature for 15 min. Samples were loaded into a 1.2% w/v agarose gel stained with 1X SYBR Safe (Invitrogen) and run at 100 V for 30 min.

2.10 Cell culture

All cell culture media and reagents were obtained from Gibco unless otherwise specified. The RAW 264.7 murine macrophage cell line (ATCC) was maintained in Dulbecco's Modified Eagle Medium High Glucose containing L-glutamine supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. The DC2.4 murine dendritic cell line (a gift from K.L. Rock, University of Massachusetts Medical School) was maintained in RPMI 1640 containing L-glutamine supplemented with 10 mM HEPES, 0.1 mM non-essential amino acids, 55 µM 2-mercaptoethanol, 10% FBS, and 1% penicillin-streptomycin. The B3Z T cell hybridoma (a gift from N. Shastri, University of California, Berkeley) was maintained in RPMI 1640 containing L-glutamine supplemented with 1 mM sodium pyruvate, 55 µM 2-mercaptoethanol, 10% FBS, and 1% penicillin-streptomycin. All cells were cultured at 37 °C and 5% CO₂.

2.11 In vitro transfection studies

RAW 264.7 or DC2.4 cells were seeded in 24-well plates at 250,000 or 200,000 cells/well in 1 mL complete medium and allowed to adhere overnight. Cells were washed with DPBS and the culture medium replaced with polyplexes or lipoplexes at 0.25 µg mRNA/well in 200 µL serum- and antibiotic-free medium. After 4 h, cells were washed and collected by PBS-based cell dissociation buffer (Invitrogen) or 0.25% trypsin-EDTA, respectively, and resuspended in DPBS containing 2% FBS and 0.2 µg/mL propidium iodide (Invitrogen). To determine GFP expression levels, 10,000 events per sample gated on viable propidium iodide negative cells were acquired on a BD FACScan flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).

2.12 Fluorescence microscopy

RAW 264.7 cells were seeded at 150,000 cells/well in 400 µL complete media in a Lab-Tek 8-well chambered coverglass (Nunc) and allowed to adhere overnight. The culture medium was replaced with polyplexes or lipoplexes at 150 ng mRNA/well in 150 µL serum- and antibiotic-free medium. After 4 h, the medium was replaced with phenol red-free medium containing 5 µg/mL Hoechst 33342 (Invitrogen). Cells were imaged on a Zeiss Axio Observer Z1 fluorescence microscope with an ApoTome optical sectioning attachment (Zeiss) using a 40x objective.

2.13 Cytotoxicity assay

RAW 264.7 cells were seeded in black 96-well plates at 50,000 cells/well in 200 μ L complete medium and allowed to adhere overnight. The culture medium was replaced with polyplexes at 50 ng mRNA/well in 40 μ L serum- and antibiotic-free medium. After 4 h, 60 μ L complete medium was added to each plate and the cells incubated for an additional 20 h. To determine cell viability based on metabolic activity, alamarBlue reagent (Invitrogen) was added directly to the culture medium at a 1:10 dilution, incubated for 2 h at 37 $^{\circ}$ C, and fluorescence measurements recorded at 585 nm.

2.14 B3Z T cell activation assay

DC2.4 cells were seeded in U-bottomed 96-well plates at 50,000 cells/well in 100 μ L complete media and allowed to adhere overnight. Polyplexes or lipoplexes were added at 50 ng mRNA/well in 100 μ L serum- and antibiotic-free media. Control cells were treated with 0.1 μ M SIINFEKL peptide (ovalbumin a.a. 257–264, GenScript). After 4 h, cells were washed and co-cultured with 100,000 B3Z cells/well in 200 μ L complete medium for 24 h. The medium was then replaced with 150 μ L/well lysis buffer containing 0.1 mM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.1% Triton X-100, and 0.15 mM chlorophenol red β -D-galactopyranoside in DPBS. After 2 h incubation at 37 $^{\circ}$ C, absorbance measurements were recorded at 570 nm.

2.15 Statistical methods

ANOVA was used to test for treatment effects and Tukey's test was used for post hoc pairwise comparisons between individual treatment groups.

3. Results

3.1 Polymer synthesis and characterization

A series of block copolymers composed of discrete DMAEMA, PEGMA, and DEAEMA-*co*-BMA segments (Table 1) were synthesized via RAFT polymerization using the general synthetic methodology outlined in Scheme 1. Triblock copolymers DPE1, PDE, and PED were designed to investigate the effect of different block arrangements without altering the lengths of their constitutive blocks. Copolymer series DE, DPE1, DPE2, and DPE3 were designed to maintain the same block order while increasing the targeted size of the central PEGMA block: 0 g/mol (DE), 10,000 g/mol (DPE1), 20,000 g/mol (DPE2), and 30,000 g/mol (DPE3). Targeted molecular weights for the DMAEMA and DEAEMA-*co*-BMA blocks in all polymers were 10,000 g/mol and 15,000 g/mol, respectively. The monomer feed ratio of 30% BMA in the DEAEMA-*co*-BMA block was selected based on our previous studies demonstrating that this composition displays membrane-disruptive properties at endosomal pH values while remaining inert under physiological conditions [37]. GPC traces of the intermediate macroCTAs and final polymer showed relatively unimodal molecular weight distributions with clear shifts to lower retention times, indicating successful block copolymerization (Fig. 1). NMR analysis confirmed that the synthesized polymers exhibited all expected resonances and were free of residual monomers (Fig. 2). All polymers exhibited narrow polydispersities ($PDI \leq 1.14$) with block sizes and compositions close to the targeted values.

3.2 pH-dependent membrane disruption

Hemolysis assays were conducted to compare the abilities of different polymer architectures to disrupt cellular membranes in a pH-dependent manner. Polymers were incubated with human red blood cells at pH values selected to mimic the acidifying gradient encountered by endocytosed material during trafficking to endosomal/lysosomal compartments (Fig. 3). All

polymers exhibited minimal hemolytic activity under physiological conditions (< 2% hemolysis at pH 7.4), but transitioned sharply to high levels of hemolysis at lower pH values. Comparing the hemolytic activities of PDE, PED, and DPE1 to assess the effect of block order on membrane destabilization activity, at pH 6.2, DPE1 and PDE achieved similar hemolysis levels of 83% and 88%, respectively, while PED exhibited only 52% hemolysis, suggesting that placing in the endosomolytic segment in the center of the polymer chain may interfere with its membrane-disruptive properties. Comparing the hemolysis levels at pH 6.2 exhibited by DE (84%), DPE1 (83%), DPE2 (79%), and DPE3 (103%), increasing the PEGMA block size in the D-P-E configuration did not alter the hemolytic profile, with the exception of DPE3 which generated markedly higher levels of hemolysis than the other three polymers. This finding suggests that sufficiently large PEGMA block lengths in the D-P-E arrangement may improve the pH-dependent membrane destabilization activity of these polymers.

3.3 Polyplex size and zeta potential

Polymers were further characterized by DLS and zeta potential measurements (Table 2). Uncomplexed polymers in solution formed particles 22–36 nm in diameter, suggestive of self-assembly into micelles and consistent with previous observations in a similar polymer system [37]. When combined with mRNA at a theoretical $\pm$ charge ratio of 4:1, all polymers completely condensed the mRNA, as confirmed by a gel retardation assay (Supplementary Data, Fig. S-1), and formed polyplexes ranging from 86–216 nm in diameter. DPE1 and PDE polyplexes were comparably sized, while PED polyplexes were significantly smaller, suggesting that the P-E-D block architecture is more favorable for compact particle formation. However, comparing mRNA polyplexes formulated from DE, DPE1, DPE2, and DPE3, increasing PEGMA block size was observed to correlate with decreasing particle size, demonstrating that improved mRNA condensation can also be achieved by lengthening the hydrophilic segment in the D-P-E configuration. The smallest particle sizes were obtained with DPE2 and DPE3, with measured polyplex diameters of $91 \text{ nm} \pm 18$ and $86 \text{ nm} \pm 16$, respectively.

Comparing PDE, PED and DPE1 to determine the effect of block arrangement on polyplex zeta potential, polyplexes formed with PDE and PED were effectively neutral while DPE1 was still slightly cationic, suggesting that having PEGMA as the middle block of the polymer chain reduces its charge-shielding capability. Comparing DE, DPE1, DPE2, and DPE3 polyplexes, increasing PEGMA size correlated with decreasing zeta potential, demonstrating that improved shielding is achieved with longer PEGMA chains, although complete charge neutralization was not achieved even with the largest PEGMA block (DPE3).

3.4 Polyplex stability

To assess the stability of the mRNA-polymer interaction, a competitive displacement assay was performed where polyplexes were incubated with increasing concentrations of the polyanion heparin (Fig. 4). Since mRNA in a polyplex does not migrate in an agarose gel, the reappearance of a free mRNA band indicates that the mRNA is no longer complexed. Polyplexes formed with PDE and PED were the least stable, with mRNA displacement beginning to be evident at heparin concentrations of 100 and 150 $\mu\text{g/mL}$, respectively. In comparison, 250 $\mu\text{g/mL}$ heparin was required to release mRNA from DPE1 polyplexes. Comparing polymer series DE, DPE1, DPE2, and DPE3, polyplexes with longer PEGMA blocks were increasingly resistant to destabilization. Polyplexes formed with DPE3, which had the largest PEGMA block, still retained some mRNA at the highest heparin concentration tested. In summary, these results indicate that the D-P-E configuration

provides the greatest polyplex stability, and that this stability is enhanced by increasing the length of the central PEGMA chain.

3.5 Evaluation of *in vitro* mRNA transfection activity

The ability of the polymers to mediate mRNA transfection *in vitro* was evaluated in two murine immune cell lines, RAW 264.7 macrophages (Fig. 5A, C) and DC2.4 dendritic cells (Fig. 5B). Cells were incubated with mRNA polyplexes for 4 h prior to analysis for GFP expression by flow cytometry. Lipofectamine 2000 (LF), a commercially available liposomal reagent, was used as a positive control. In both cell lines, PDE and PED polyplexes failed to produce significant mRNA transfection (< 2% GFP+ cells). Polyplexes formed with DE, the diblock copolymer lacking a PEGMA block, generated 8% GFP+ cells, much lower than the efficiency obtained with LF (30%). In contrast, DPE1, DPE2, and DPE3 polyplexes were very efficient at transfecting mRNA, with GFP expression levels much higher than LF. In RAW 264.7 cells, the transfection efficiency increased dramatically with increasing PEGMA block size: 38% (DPE1) vs. 68% (DPE2) vs. 77% (DPE3). This trend was also observed in DC2.4 cells, but was less pronounced: 43% (DPE1) vs. 45% (DPE2) vs. 50% (DPE3). Overall, these findings demonstrate the importance of polymer architecture in successful mRNA transfection: polymers with the D-P-E arrangement were the most effective for intracellular mRNA delivery and were capable of mediating transfection levels superior even to LF, while polymers with alternative block arrangements produced very low gene expression.

3.6 Polyplex cytotoxicity

The cytotoxicity of the mRNA polyplexes was evaluated by an alamarBlue assay using RAW 264.7 macrophages (Fig. 6). Cells were incubated with polyplexes for 24 h, after which cell viability was quantified based on total metabolic activity resulting in reduction of the alamarBlue reagent. All polyplexes exhibited low cytotoxicity levels (> 75% viability). The trends in cell viability correlated closely to transfection activity levels, with higher toxicity observed with polymers that also exhibited greater mRNA delivery efficacy.

3.7 Antigen presentation assay

To determine whether dendritic cells transfected with mRNA polyplexes could successfully process and present the encoded antigen on MHC molecules, an *in vitro* MHC I antigen presentation assay was performed using the B3Z CD8+ T cell hybridoma (Fig. 7). The B3Z cell line produces β -galactosidase upon recognition of the ovalbumin CD8 epitope (SIINFEKL) presented in the context of MHC I H-2K^b [41]. DC 2.4 cells were transfected with polyplexes containing ovalbumin mRNA for 4 h, then cocultured with B3Z cells for 24 h, and the overall T cell response determined by assaying for β -galactosidase activity. The resultant T cell responses were normalized relative to a peptide positive control (0.1 μ M SIINFEKL).

All polymers except for PDE and PED generated high levels of T cell activity, outperforming LF. DPE1 in particular produced the highest T cell response, despite having a slightly lower mRNA transfection efficiency than DPE2 and DPE3. Similarly, DE produced less gene expression than LF in the transfection assay, but mediated higher levels of T cell activity. In summary, these findings demonstrate that mRNA transfection mediated by these polymer systems can lead to antigen presentation and subsequent T cell activation, although the level of activation does not necessarily correlate precisely to the efficiency of transfection.

4. Discussion

Although multifunctional ABC-type triblock copolymer systems have been previously investigated for pDNA and siRNA transfection, to the best of our knowledge this is the first report of a triblock copolymer developed specifically as an mRNA delivery vector. Our triblock system was composed of a DMAEMA block for cationic mRNA condensation, a PEGMA block to provide hydrophilic stabilization, and DEAEMA-*co*-BMA to enable pH-responsive endosomal escape.

Typically, triblock copolymer architectures incorporating a hydrophilic segment for steric stabilization place this block at the chain end. Accordingly, two of the polymers in this study, PDE and PED, were synthesized with PEGMA at one end and either DMAEMA or DEAEMA-*co*-BMA at the other. A third polymer, DPE1, was synthesized with the same block lengths as PDE and PED, but placed the PEGMA block in the middle of the polymer chain instead. Intriguingly, of these three polymers, only DPE1 was capable of mediating mRNA transfection and antigen presentation *in vitro*. To further explore this phenomenon, the effect of PEGMA block size was investigated by synthesizing a series of triblock copolymers in the D-P-E configuration with increasing PEGMA length (DPE1, DPE2, DPE3), as well as a diblock copolymer lacking PEGMA (DE). Transfection studies demonstrated that delivery efficacy improved significantly with increasing PEGMA block size, though the magnitude of the difference was dependent on cell type. The most efficient polymers were able to surpass a commercial liposomal reagent, Lipofectamine 2000, in mRNA transfection activity.

Heparin displacement studies suggested that the differences in mRNA transfection were potentially linked to polyplex stability: the most stable polyplexes were also those that exhibited the highest transfection efficiencies. Destabilization of polyplexes by anionic compounds such as serum proteins, extracellular matrix components, and cell surface glycosaminoglycans constitutes a significant barrier to intracellular gene delivery [42–45]. While PEGylation improves polyplex stability by creating a hydration shell that masks excess cationic charges [12,44,45], hydrophilic polymers may also interfere with nucleic acid condensation [46,47]. Of the six polymer systems investigated in this report, polyplexes formulated with PDE and PED were also the least stable to heparin displacement, despite having near-neutral zeta potentials indicative of complete hydrophilic shielding. In contrast, polymers in the D-E and D-P-E arrangements exhibited improved stability, with longer PEGMA block lengths conferring stronger mRNA binding and reduced cationic surface charge. The D-P-E polymer architecture in particular appeared to achieve an optimal balance between charge shielding and polyplex stability that translated into highly efficient mRNA transfection.

While Üzgün *et al.* also concluded that improved mRNA binding led to improved transfection [18], other groups have demonstrated that highly stable mRNA-polymer interactions can be detrimental to transfection, presumably due to the inability of these vehicles to release mRNA in the cytosol [14,15,19]. These findings are not necessarily inconsistent, given the structural and compositional differences between the polymer systems used in these various studies. Taken together, it is clear that polyplex stability is an important factor that needs to be carefully modulated in order to achieve optimal mRNA delivery.

To determine whether efficient mRNA transfection translated to successful antigen presentation and subsequent T cell activation, DC2.4 cells transfected with mRNA polyplexes were cocultured with ovalbumin-specific CD8⁺ T cells. All polymers except for PDE and PED generated T cell responses that surpassed those attained by Lipofectamine

2000. Surprisingly, polymers DE and DPE1 produced greater levels of T cell activation than might be expected from their relative transfection levels. DPE1 notably produced the highest T cell response, despite exhibiting transfection efficiencies in DC2.4 cells comparable to DPE2 and DPE3. These discrepancies may potentially be linked to differences in polyplex size, as DPE1 polyplexes were observed to be significantly larger than DPE2 and DPE3 polyplexes. Previous reports have observed that carrier size can influence the efficiency of intracellular antigen trafficking and processing for protein vaccines [48,49], and similar mechanisms may also be important for nucleic acid vaccination. Overall, the positive results obtained with the D-P-E polyplexes in the antigen presentation assay demonstrate that this system is a promising candidate for the delivery of mRNA-based vaccine agents.

5. Conclusions

mRNA is a promising gene vaccine agent, but clinical development is hindered by the dearth of research efforts towards the development of safe, efficient delivery vehicles. Here, a series of multifunctional block copolymers composed of discrete DMAEMA, PEGMA, and DEAEMA-co-BMA segments with systematically varied polymer architectures were synthesized by RAFT polymerization and evaluated for their ability to facilitate intracellular mRNA delivery. Polymers exhibited pH-dependent hemolytic activity, favorable size and charge when complexed with mRNA, and low polyplex cytotoxicity. The D-P-E architecture with the PEGMA block in the center of the polymer chain demonstrated enhanced polyplex stability against heparin displacement relative to other configurations, with longer PEGMA block lengths enhancing mRNA binding. This system achieved superior mRNA transfection efficiencies and high levels of antigen-specific T cell activation *in vitro*, demonstrating its potential as a gene delivery vehicle for mRNA-based vaccination strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Bilal Ghosn and Debashish Roy for their assistance in protocol development for the RAFT synthesis of PEGMA. This work was supported by the National Institutes of Health (R01AI074661). C.C. is supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-0718124 and the Department of Defense through the National Defense Science and Engineering Graduate Fellowship Program.

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Figure 1. Representative molecular weight distributions obtained by gel permeation chromatography for intermediate macroCTAs and final triblock copolymer (DPE1) synthesized via RAFT polymerization.

Figure 2. Representative $^1\text{H-NMR}$ (CDCl_3) spectrum of a triblock copolymer (DPE1). Monomer compositions were determined by integration of peaks i, vi, xxi, and (iii, x, xvi, xxii).

Figure 3.

Hemolytic activity of polymers at 20 $\mu\text{g/mL}$ mass concentration of the DEAEMA-*co*-BMA block. Polymers were incubated with red blood cells at the indicated pH values for 1 h at 37 $^{\circ}\text{C}$. Hemolytic activity was quantified based on absorbance measurements for hemoglobin release. Activities are normalized relative to a Triton X-100 positive control and DPBS negative control and are from a single representative experiment conducted in triplicate $\pm$ standard deviation.

Figure 4. mRNA polyplex stability against heparin displacement. Polyplexes were incubated with equal volumes of heparin solutions at the indicated concentrations for 15 min at room temperature prior to agarose gel electrophoresis.

Figure 5.

In vitro transfection efficiencies in (A) RAW 264.7 macrophages and (B) DC2.4 dendritic cells. Lipofectamine 2000 (LF) was used as a positive control. Cells were treated with mRNA polyplexes for 4 h, then analyzed by flow cytometry for GFP expression. Data are from a single representative experiment conducted in triplicate $\pm$ standard deviation. In (A), DPE2 and DPE3 are significantly different than all other treatments and each other ($p < 0.05$). In (B), DPE1, DPE2, and DPE3 are significantly different than all other treatments, and DPE3 is significantly different than DPE1 ($p < 0.05$). (C) Fluorescence micrograph of GFP expression in RAW 264.7 macrophages (blue, Hoechst nuclear stain) 4 h post-transfection. Scale bar is 50 μ m.

Figure 6.

In vitro cytotoxicity of polyplexes in RAW 264.7 macrophages. Cells were treated with mRNA polyplexes for 24 h prior to cell viability analysis via alamarBlue (Invitrogen) assay. Viabilities are normalized relative to untreated cells. Data are from a single representative experiment conducted in triplicate $\pm$ standard deviation.

Figure 7.

B3Z T cell activation by transfected DC2.4 dendritic cells. DC2.4 cells were treated with mRNA polyplexes encoding ovalbumin for 4 h, then cocultured with B3Z T cells for 24 h. T cell activation was then quantified based on β -galactosidase activity. Responses are normalized relative to cells treated with 0.1 μ M SIINFEKL peptide as a positive control and are from a single representative experiment conducted in triplicate $\pm$ standard deviation. DPE1, DPE2, and DPE3 are significantly different than LF, and DPE1 is significantly different than all other treatments ($p < 0.05$).

Scheme 1.

RAFT-mediated synthesis of a D-P-E triblock copolymer composed of discrete cationic DMAEMA, hydrophilic PEGMA, and endosomolytic DEAEMA-*co*-BMA segments.

Table 1

Blocking orders, molecular weights, polydispersities, and monomer compositions for polymer designs.

Polymer	Block order ^a	M _n 1st block ^b (g/mol)	DP 1 st block ^b	M _n 2 nd block ^b (g/mol)	DP 2 nd block ^b	M _n 3 rd block ^b (g/mol)	DP 3 rd block ^b	Total M _n ^b (g/mol)	PD ^b	%BMA E block ^c
DE	D-E	11600	74	13500	79	-	-	25100	1.07	34
DPE1	D-P-E	11600	74	8400	28	15500	90	35500	1.08	31
DPE2	D-P-E	11600	74	19700	66	14300	84	45600	1.12	36
DPE3	D-P-E	11600	74	33100	110	17100	100	61700	1.14	34
PDE	P-D-E	10900	36	10300	66	13100	77	34400	1.08	34
PED	P-E-D	10900	36	16200	95	8100	52	35200	1.11	33

^a Denotes the ordering of the DMAEMA (D), PEGMA (P), and DEAEEMA-co-BMA (E) blocks (1st block-2nd block-3rd block).

^b As determined by GPC.

^c The monomer composition of the DEAEEMA-co-BMA (E) block, as determined by ¹H-NMR (CDCl₃) spectroscopy (Bruker AV 500).

Table 2

Particle sizes and zeta potential measurements of free polymer micelles and mRNA polyplexes.

Polymer	Polymer micelle diameter (nm)	mRNA polyplex diameter (nm)	mRNA polyplex zeta potential (mV)
DE	25 ± 2.4	201 ± 38	21.0 ± 0.5
DPE1	29 ± 3.1	208 ± 51	20.3 ± 0.7
DPE2	32 ± 3.9	91 ± 18	16.3 ± 1.0
DPE3	36 ± 3.9	86 ± 16	12.4 ± 0.7
PDE	33 ± 5.5	216 ± 66	3.6 ± 0.7
PED	22 ± 2.9	106 ± 30	3.4 ± 0.4

Diameters are calculated from the intensity size distribution ± standard deviation calculated from the PDI. Zeta potential values are from three experimental runs ± standard deviation.