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Delivery of antisense oligonucleotides using poly(alkylene oxide)-poly(propylacrylic acid) graft copolymers in conjunction with cationic liposomes

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Abstract

The clinical application of gene silencing is hindered by poor stability and low delivery efficiency of naked oligonucleotides. Here, we present the *in vitro* and *in vivo* behavior of a rationally designed, ternary, self-assembled nanoparticle complex, consisting of an anionic copolymer, cationic DOTAP liposome, and antisense oligonucleotide (AON). The multifunctional copolymers are based on backbone poly (propylacrylic acid) (PPAA), a pH-sensitive hydrophobic polymer, with grafted poly (alkylene oxides) (PAOs) varying in extent of grafting and PAO chemistry. The nanoparticle complexes with PPAA-g-PAO copolymers enhance antisense gene silencing effects in A2780 human ovarian cancer cells. A greater amount of AON is delivered to ovarian tumor xenografts using the ternary copolymer-stabilized delivery system, compared to a binary DOTAP/AON complex, following intraperitoneal injection in mice. Further, intratumoral injection of the nanoparticle complexes containing 1 mol% grafted PAO reduced tumoral bcl-2 expression by up to 60%. The data for complexes across the set of PAO polymers support a strong role for the hydrophilic-lipophilic balance of the graft copolymer in achieving serum stability and cellular uptake. Based upon these results, we anticipate that this novel nanoparticle delivery system can be extended to the delivery of plasmid DNA, siRNA, or aptamers for preclinical and clinical development.

Graphical abstract

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Keywords

antisense; gene silencing; nanoparticle; drug delivery; self assembly; tumor

1. Introduction

Antisense oligonucleotides (AONs) are under development for disease states such as cancer that are defined by abnormal gene expression [1–3]. However, after administration AONs encounter multiple systemic and cellular delivery barriers. Naked oligodeoxynucleotides are cleared by renal filtration and, unless chemically modified, are degraded within minutes by nucleases in the bloodstream [4]. The use of nanocarriers for nucleic acid delivery increases AON half-life from minutes to hours [5]. The progress for antisense as a therapy is dependent on the development of an AON delivery system that will mediate effective intracellular delivery and transport effective doses of AON to the target disease site. To achieve this goal, chemically diverse carriers, primarily in the form of nanoparticles, are being formulated and evaluated.

Materials used as carriers for nucleic acid delivery include liposomes, polymers, micelles, and biological macromolecules [6–10]. The chemistry of liposomes is appealing as they are naturally amphiphilic, therefore promoting interaction with cell membranes. Unfortunately, conventional liposomes generally require optimized coverage with poly(ethylene oxide) for serum stability and circulation half-life, and their chemical space and ease of functionalization are limited [11, 12]. On the other hand, polymers such as poly(L-lactic acid), polyethyleneimine and poly(amidoamine) have been explored for nucleic acid delivery. The advantage of using polymers is the ease of manipulating their chemistry to incorporate functional groups including pH-sensitive polymers and targeting ligands [13–21]. Cationic polymers, however, can be cytotoxic and non-biodegradable [15, 22–24]. We and others have attempted to combine the advantages of liposomes and polymers in a single nanocomposite delivery system toward the goal of attaining maximal antisense delivery efficiency, while minimizing undesired cytotoxic effects [25–28].

The physical properties of nanoparticles such as their size, charge, and chemistry affect their intracellular gene delivery characteristics and systemic properties including blood residence

time, ability to localize to tumors through the enhanced permeation retention (EPR) effect, and rate of clearance by liver, spleen or kidney. While the effects of particle size and charge on nucleic acid delivery have been explored extensively, the effect of hydrophilic-lipophilic balance (HLB) has been comparatively less studied [29–34]. Wang et al. investigated amphiphilic modification of chitosan by varying the proportions of hydrophobic linoleic acid and hydrophilic poly β -malic acid, finding an optimum degree of hydrophobicity (lipophilicity) where maximum association with RBC model membranes and cellular uptake was observed [35]. Kabanov et al. have introduced Pluronics[®], triblock copolymers of poly(ethylene oxide), PEO, and poly(propylene oxide), PPO, for drug and gene delivery applications [36, 37] and identified HLB as an important parameter in the activity of Pluronics[®] formulations [38, 39]. These studies suggest that a deeper understanding of the effects of polymer HLB on delivery vector function would enable the design of improved cell-interactive biomaterials for drug and nucleic acid delivery.

We have previously reported the development of a novel ternary nanoparticle complex of graft copolymer-liposome-polynucleotide that was designed to overcome the intracellular barrier of endosome degradation and the extracellular barrier of serum instability [27]. The copolymers, comprised of a pH-sensitive backbone of poly (propyl acrylic acid) grafted with either hydrophilic PEO or amphiphilic poly(ether amine), were designed to impart resistance to serum protein attack, cellular association, and endosomal escape. The ternary nanocomplex was found to self-assemble into nanoparticles of 200–300 nm in size and to mediate effective gene silencing in cell culture with serum-containing media. In this study, we investigated the effect of the graft copolymer HLB on AON delivery by synthesizing and evaluating copolymers with varying graft densities of PEO or poly(ether amine) chains. *In vitro* AON delivery functions of the nanoparticle complexes were evaluated by studying membrane penetration, membrane lysis, and gene silencing of the bcl-2 gene in serum-containing cultures of human ovarian cancer cells. *In vivo* studies were conducted to determine the nanoparticles' biodistribution of the antisense AON and its gene silencing activity in human ovarian cancer xenografts, which was indicative of the therapeutic relevance of the delivery system for cancer chemotherapy.

Materials and Methods

1.1. Synthesis and characterization of PAO graft copolymers

N,N'-Diisopropylcarbodiimide (DIPC), 1-Hydroxybenzotriazole hydrate (HOBt), tetrahydrofuran (THF) anhydrous and dimethyl sulfoxide (DMSO) were obtained from Aldrich. Jeffamine[®] polyetheramines, which are copolymers of ethylene oxide (EO) and propylene oxide (PO), were kindly provided by Huntsman, The Woodlands, Texas. Jeffamine[®]M-2070 and Jeffamine[®]M-2005 ($M_n=2000$, monoamine) with EO to PO ratios of 31/10 and 6/29, respectively, were used. Poly (propyl acrylic acid) (PPAA, $M_n=200$ kDa) was obtained from Polymer Source, Montreal, Canada. Pierce Dialyzer cassette was obtained from Thermo Scientific, Rockford, IL. Amine-terminated mPEG-NH₂ was obtained from creative PEG works, Winston Salem, NC.

1.1.1. Synthesis of poly (alkylene oxide) (PAO) graft copolymers (Scheme 1)—

PPAA (50 mg, 0.44 mol of repeat units) was taken in a dry 10 mL round bottomed flask. HOBt (9 mg, 0.044 mmol) and DIPC (10.2 μ L, 0.044 mmol) were added to the flask. Dry THF (5 mL) was added to the flask and the reaction was stirred at room temperature for 45 min. After this, amine-terminated Jeffamine or PEO (0.0044, 0.022, 0.044 moles corresponding to 1, 5, 10 mol% target graft densities, respectively) was added and the reaction was stirred at 50°C for 30 h. Scheme 1 outlines the chemical reaction of PPAA with amine-terminated PEO, Jeffamine[®]M-2070 and Jeffamine[®]M-2005. After the reaction time, THF was removed and dried product was resuspended in a solution of 10% by volume 1 N NaOH and 90% by volume PBS, pH 7.4. This mixture was transferred to a Pierce Dialyzer cassette (MWCO 10000 Da) and dialyzed against distilled water for 3 days, with three buffer changes in the first 6 h, followed by one buffer change every 12 h for the remaining time period. The product was then removed, lyophilized and characterized by proton NMR using DMSO as the solvent.

2.1.2. Characterization of PAO graft copolymers—Using the amide peak for NMR integration is the most direct and accurate method to determine the degree of conjugation, however, since the peak is very small for integration, this method is inaccurate when using small quantities of material for analysis. Hence, the following methods have been used: 1) Jeffamine M2070 graft copolymers: the peaks of CH₃ on PPAA and CH₃ on Jeffamine. 100% grafting is indicated by a 1:10 ratio of CH₃ on PPAA and CH₃ on propylene oxide of Jeffamine, respectively 2) Jeffamine M2005 graft copolymers: the peaks of CH₃ on PPAA and CH₃ on Jeffamine. 100% grafting is indicated by a 1:29 ratio of CH₃ on PPAA and CH₃ on propylene oxide of Jeffamine, respectively 3) PEO graft copolymers: the peaks of H on CH₃ of PPAA and H on EO of PEO. 100% grafting is indicated by a 3:180 ratio of H on CH₃ of PPAA and H on CH₃ of EO, respectively. For PPAA-g-1mol%Jeffamine M2070: ¹H NMR (DMSO-d₆) 0.8 (s, CH₃), 1 (C-CH₃), 3.5 (CH₂, Jeffamine) are 3, 0.98, 2.8, respectively. For PPAA-g-5mol%Jeffamine M2070: ¹H NMR (DMSO-d₆) 0.8 (s, CH₃), 1 (C-CH₃), 3.5 (CH₂, Jeffamine) are 3, 2.38, 8.31, respectively. For PPAA-g-10mol %Jeffamine M2070: ¹H NMR (DMSO-d₆) 0.8 (s, CH₃), 1 (C-CH₃), 3.5 (CH₂, Jeffamine) are 3, 3.3, 14.23, respectively. For PPAA-g-1mol%Jeffamine M2005: ¹H NMR (DMSO-d₆) 0.8 (s, CH₃), 1 (C-CH₃), 3.5 (CH₂, Jeffamine) are 3, 1.87, 2.01, respectively. For PPAA-g-1mol%PEO: ¹H NMR (DMSO-d₆) 0.8 (s, CH₃), 3.3–3.5 (–CH₂CH₂O–) are 3, 2.63, 3.91, respectively. For PPAA-g-5mol%PEO: ¹H NMR (DMSO-d₆) 0.8 (s, CH₃), 3.3–3.5 (–CH₂CH₂O–) are 3, 6.31, 16.23, respectively. For PPAA-g-10mol%PEO: ¹H NMR (DMSO-d₆) 0.8 (s, CH₃), 3.3–3.5 (–CH₂CH₂O–) are 3, 10.32, 31.71, respectively.

2.2. Calculation of hydrophilic-lipophilic balance (HLB)

The calculations of rHLB were made on a molar basis. The graft copolymers synthesized are anionic conjugates containing hydrophilic and hydrophobic components. HLB values were calculated using group numbers of prominent hydrophilic (carboxylic acid, ethylene oxide) and hydrophobic groups (propylene oxide). Note that the contribution of the backbone PPAA was not included because it is the same for all graft copolymers. To establish a range from 0 to 1, these values were normalized to PPAA (control, unmodified polymer) and

PPAA-g-10%PEO (most hydrophilic polymer). The calculation of HLB is as follows, and the values for each graft copolymer are summarized in Table 1:

$$rHLB = \frac{H(\text{graft copolymer}) - H(\text{PPAA})}{H(\text{PPAA-g-10\%PEO}) - H(\text{PPAA})} \quad (1)$$

$$H(\text{graft copolymer}) = \sum \alpha * n_1(\text{COOH}) + \beta * n_2(\text{EO}) + \gamma * n_3(\text{PO}) \quad (2)$$

$$H(\text{PPAA}) = \sum \alpha * n_4(\text{COOH}) \quad (3)$$

H =hydrophilicity, n_i =number of each group per copolymer, EO=ethylene oxide, PO=propylene oxide, COOH=carboxylic acid, $\alpha=2.1$, $\beta=0.35$, $\gamma=-0.125$. Values from [40].

2.3. Hemolysis assay

The ability of control polymer, PPAA, and the grafted copolymers to disrupt membranes was assessed using a hemolysis assay as described previously [27]. RBCs were obtained from Rockland (Gilbertsville, PA, USA) and used within two days of isolation. Briefly, solutions of PPAA and graft copolymers at equivalent moles of carboxylic acid groups were formulated in buffers of pH values ranging from 5.0 to 7.0. Freshly washed red blood cells (RBCs) were added at a concentration of 10^8 cells/mL, incubated in a water bath at 37°C for 1 h, and then centrifuged for 4 min at 400 g to pellet the intact cells. The absorbance of the supernatant, consisting of RBC lysate, was measured at 540 nm using a UV spectrophotometer (Thermo Spectronic) and normalized to conditions consisting of RBCs in equivalent buffers in the absence of polymer (negative control) and RBCs in distilled water (positive control).

2.4. Calcein dye leakage from dye-loaded DPPC liposomes

Dipalmitoylphosphatidylcholine (DPPC) liposomes were prepared following the general protocol provided by Avanti Polar Lipids. Briefly, a thin lipid film was obtained by using a rotary evaporator to dry the lipid solution, followed by hydration of the lipid film with calcein dye by agitation and heating/stirring at 50°C for 2 h (Note: this temperature is greater than the phase temperature of the lipid). The weight ratio of DPPC lipid to calcein was 1:3 to ensure full loading of dye. Further, the samples were agitated and sonicated for 15 minutes to form lamellar vesicles, followed by 5 cycles of freeze/thaw and extrusion using a 100 nm polycarbonate membrane. Finally, unloaded calcein dye was separated from DPPC liposomes using a Sephadex column.

For the membrane penetration assay, varying amounts of PPAA and graft copolymers were mixed with 0.68 mM calcein loaded in DPPC liposomes and allowed to incubate for 1 h at 37°C in a pH 7.4 buffer solution. Subsequently, fluorescence resulting from release of calcein dye was measured at an excitation wavelength of 490 nm and emission of 520 nm. Values of fluorescence were normalized to a positive control consisting of dye-loaded DPPC liposomes incubated with Triton X-100 and a negative control consisting of dye-loaded

DPPC in the absence of polymer or surfactant. Because PPAA and graft copolymers vary in molecular weight, their amounts were controlled in regard to the number of propylacrylic acid groups, i.e. –COOH groups, present.

2.5. Nanoparticle preparation and cell culture evaluation

A2780 human ovarian cancer cells were obtained from American Type Culture Collection. Cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Nanocomplexes were prepared as described previously [27], utilizing a two-step procedure in which AON and DOTAP are initially incubated for 30 min. to form binary complexes, and subsequently anionic polyelectrolyte is added with an additional 30 min. incubation.

Briefly DOTAP (*N*-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl-sulfate) liposome at a stock concentration of 1 mg/ml was diluted in PBS and added to AON (stock concentration of 1 µM) also diluted in PBS to achieve a charge ratio of 4.7. The DOTAP and AON were allowed to incubate for 30 min. PPAA and its derivatives were all solubilized in 10% by volume of 1 M NaOH and 90% by volume of PBS (pH 7.4) to prepare 10 mg/ml polymer solutions. From the stock of 10 mg/ml, dilutions to 1 mg/ml were made by using PBS. Next, PPAA (stock concentration of 1 mg/ml) was titrated in to achieve a final net charge ratio of 1, and the final complex of DOTAP/ODN/PPAA was allowed to form via electrostatic interactions for an additional 30 min. All the components were pipette mixed and not vortexed. The volume ratio of DOTAP to AON to PPAA was 1:1:0.5 for a total of 150 µl. The final complex was then added to cells containing 10% FBS-containing media (of 850 µl) for each well of a 12-well plate and allowed to be taken up by cells for 4 hours.

All of the complexes were prepared using a DOTAP/AON charge ratio (+/-) of 4.7 and a DOTAP/AON/Polymer net charge ratio of 1.0, where the net charge ratio is defined as the ratio of the moles of DOTAP amine groups to the sum of the moles of AON phosphate groups and PPAA carboxylic acid groups, with the latter assumed to be 33% ionized at pH 7.4. DOTAP liposomal transfection reagent was obtained from Roche Applied Science (Indianapolis, IN, USA).

2.5.1. In vitro cell metabolic activity—Cells were cultured on 96-well plates and treated with the various graft copolymer formulations at 2 µg/mL AON concentration. The AON sequence used was complementary to rabbit β-globin and is not complementary to any human gene. Cell metabolic activity was determined using the MTS assay at various time points (48, 72 and 96 h post-treatment) using the CellTiter 96® AQueous Non-radioactive Cell Proliferation (MTS) kit (Promega, Madison, WI, U.S.A).

2.5.2. In vitro gene silencing—A2780 cells were seeded onto 12-well plates at 10⁵ cells/mL (with 1 mL volume per well) ~18 h prior to AON treatment. All AON sequences were obtained from IDT Technologies (Coralville, IA) and delivered as HPLC grade. The lyophilized AONs were resuspended to 500 µM concentration using TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The bcl-2 AON sequence (5'-TCTCCCAGCGTGCGCCAT-3' [41]) and scrambled control (5'-TTGCTTGTACCGTGCGTGCC-3') was used at a concentration of 300 nM. Complexes were prepared in a volume of 200 µL, followed by

mixing with 10% FBS-containing medium and addition to each well. For control samples, complexes were substituted with 200 μ L of PBS.

After 4 hours of exposure, medium containing complexes was aspirated and replaced with fresh medium. Cells were assayed for bcl-2 gene expression 24 h post-AON treatment using real-time PCR, with 18S ribosomal RNA as the internal standard. The Ct method was used to calculate % bcl-2 gene expression [42].

2.6 In vivo evaluation

Female athymic nude mice of age 6–8 weeks (~ 20 g) were purchased from Charles River Laboratories (Wilmington, MA). All work performed on animals was in accordance with and permitted by the Rutgers University Institutional Animal Care and Use committee. The bcl-2 AON sequence was labeled with Cy5.5 fluorescence tag and used for biodistribution studies. The same sequence without fluorescence tag and scrambled control were used. For intratumoral gene silencing studies, the bcl-2 AON sequence (no tag) and scrambled control were used. AON of HPLC grade was obtained from IDT technologies and solubilised in TE buffer at 1 μ M concentration.

2.6.1. Animal tumor model—An animal model of human ovarian carcinoma xenografts was created as previously described [43–45]. Briefly, A2780 human ovarian cancer cells were passaged at 70% cell density, during their peak growth phase. At this point, $6\text{--}8 \times 10^6$ cells were transplanted subcutaneously into the flanks of athymic nude mice (of 8 weeks age). The tumor size was measured with a caliper and its estimated volume was calculated as $d^2 \times D/2$, where d and D are the minor and major axes, respectively, of the roughly elliptically-shaped tumor surface.

2.6.2. Biodistribution—When the tumor reached a size of about 400 mm³ (15–25 days after transplantation), 250 μ L of the various formulations containing 0.625 mg Cy5.5-AON/kg were administered to the mice by intraperitoneal (i.p.) injection. After 24 h, mice were sacrificed, and their tissues were collected and imaged by the IVIS Imaging System (Xenogen, Alameda, CA). All fluorescence values were adjusted by subtracting autofluorescence signal from tissues collected from mice treated with PBS. Organs (liver, kidney, heart, spleen, lung and tumor) were excised and weighed for normalization.

2.6.3. Bcl-2 intratumoral gene silencing—Silencing of the bcl-2 gene in tumors was measured 24 h after mice were treated by intratumoral injection of 250 μ L of the various formulations containing 0.625 mg of the antisense AON/kg. Following treatment, each tumor was excised, weighed, and homogenized using a tissue homogenizer. The mRNA from cells was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized using Ambion RETROscript® Kit (Invitrogen, Carlsbad, CA). Using the cDNA, the rt-PCR reaction was conducted to amplify 18S and bcl-2 and detected using QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA). Bcl-2 gene expression in treated mice was normalized to that of untreated animals.

2.7. Statistical analysis

All statistical analyses were performed by a one-way ANOVA test with Tukey post-hoc pairwise comparison test. Data was considered statistically significant when p value was less than 0.05. All analyses were performed using Kaleidagraph v4.

3. Results

3.1. Synthesis of PAO graft copolymers

Graft copolymers with varying degrees of hydrophilic-lipophilic balance were synthesized by modifying poly(alkylene oxide) (PAO) graft chemistry and degree of PAO graft density onto PPAA. Specifically, in order of decreasing hydrophilicity, the three graft chemistries included the most hydrophilic, PEO, followed by Jeffamine M-2070 (ethylene oxide (EO) to propylene oxide (PO) of 3 to 1) and the most lipophilic, Jeffamine M-2005 (EO/PO= 1/6). For each graft chemistry, polymers of 1, 5 and 10 mol% target graft densities onto the PPAA backbone were synthesized. NMR was performed after ensuring that all the unconjugated PEO/Jeffamine was removed by exhaustive dialysis. The experimental graft density of each copolymer was calculated using CH₂-CH₂-O peaks of PEO- and -OCH₃ peaks of Jeffamine, relative to CH₂CH₃ peaks of PPAA. An alternative method involves quantifying the amide peaks of the graft copolymer relative to the propyl peaks of PPAA; however, this method was not applied because the size of the amide peak was very small (even when several mg of polymer were used). Table 1 summarizes the various graft copolymers synthesized, their targeted graft densities and experimental graft densities determined by NMR, their solubility characteristics, and their hydrophilic-lipophilic balance (HLB) values. The HLB values were normalized to PPAA (value of 0) and the most hydrophilic copolymer, PPAA-g-10%PEO (value of 1). Copolymers with greater than 1% (nominal) grafting of the PO-rich Jeffamine M-2005 proved to be insoluble in aqueous media and were therefore not included for further experimentation.

3.2. Hemolysis induced by PPAA and PPAA graft copolymers

PPAA has garnered attention for drug and nucleic acid delivery based on its ability to exhibit pH-dependent hemolysis, which has been associated with the ability to escape the low pH (~5) endolysosomal pathway within cells [46]. We determined the effect of PAO grafting on this property. Only the ungrafted PPAA and the graft copolymers with 1% (theoretical) graft densities of PEO, Jeffamine-M2070 and Jeffamine M-2005 caused hemolysis (Fig. 1).

Higher graft densities of 5 and 10 % eliminated the lysis effect entirely over the pH range from 5.0 to 7.0 (conditions not shown). The copolymers formed by 1% grafting of PEO or Jeffamine-M2070 exhibited the hemolysis maximum at pH 6.0 that is well established for PPAA [46, 47], with greatly reduced hemolysis at both higher and lower pH values. The maximum hemolysis activity at pH 6.0 was only minimally decreased by grafting PPAA with 1% PEO but was more significantly decreased by grafting PPAA with 1% Jeffamine M-2070. In contrast, the most hydrophobic graft copolymer, 1% Jeffamine M-2005, was most hemolytic at pH 7.0.

3.3. Membrane activity

The ability of a carrier to penetrate or fuse through membranes has been characterized by measuring calcein dye leakage from dye-loaded DPPC liposomes [48–50]. We have found that this property correlates with AON-mediated gene silencing in cell culture [51]. Calcein dye leakage was measured across a set of polymer concentrations (indicated in terms of their number of carboxyl groups) in the sensitive range. The release of calcein decreased steadily as the grafting density of Jeffamine M-2070 (Fig. 2A) or PEO (Fig. 2B) was increased.

PPAA-g-1% Jeffamine M-2005 displayed 100% calcein release throughout the range of concentrations tested (data not shown), consistent with the fact that this polymer was more hydrophobic than any of the other polymers. Overall, decreasing calcein release was associated with increasing graft density of both polymer types (PPAA-PEO and PPAA-Jeffamine). At some graft densities, the Jeffamine containing polymers induced slightly more calcein release than their PEO containing counterparts, but overall the influence of graft type was minor.

3.4. Effect of DOTAP/AON/Polymer treatment on A2780 cell metabolic activity

The first biological question addressed was whether the HLB of the PAO graft copolymers has any effect on cytotoxicity in cell culture. In accordance with *in vitro* silencing protocols, nanocomplexes were added to A2780 ovarian cancer cells for 4 h, followed by measurement of metabolic activity at 48 h post-treatment. The cellular metabolic activity indicates that the most hydrophobic polymer, PPAA-g-1%Jeffamine M 2005 ('Pg1%J05'), was significantly more cytotoxic than the control or other graft copolymers (Fig. 3). All the other graft copolymer systems displayed between 90–100% cell metabolic activity (Fig. 3). The data suggest that the cytotoxicity induced by the carrier is related to carrier hydrophobicity, since cytotoxicity becomes negligible for polymers with increasing graft density, i.e. increasing hydrophilicity. The PPAA-g-1%Jeffamine M 2005 condition was eliminated from the panel of graft copolymers because of its cytotoxic effects with RBCs and with A2780 cells (Figs. 1 and 3), whereas for all other carriers the cytotoxicity was considered to be within acceptably low limits.

3.5. Bcl-2 gene silencing in cell culture

The ability of graft copolymer complexes to deliver AON in serum-containing cell cultures and to silence the bcl-2 gene was evaluated using the A2780 human ovarian carcinoma cell line. The expression of bcl-2 gene was evaluated 24 h post-treatment, which provides sufficient time for cellular internalization of AON and turnover of existing mRNA [52]. The binary complex of DOTAP/AON induced only modest gene silencing, with 80% bcl-2 gene expression relative to that of untreated cells (Fig. 4A). The addition of PPAA to DOTAP/AON complexes improved the antisense gene silencing effect, reducing the bcl-2 expression to 40% of the untreated control. This enhanced silencing effect caused by the addition of PPAA has also been observed using a CHO-d1EGFP cell line [27]. The ternary nanoparticle complexes with the lowest graft density copolymers (1% Jeffamine M 2070 and 1% PEO) further improved antisense gene silencing, resulting in 10 and 15% bcl-2 gene expression levels, respectively (Fig. 4A). However, the 5 and 10 % graft density Jeffamine and PEO copolymers progressively decreased gene silencing, with bcl-2 gene expression

levels similar to control, untreated cells (Fig. 4A and B). Some non-specific silencing was observed when an irrelevant sequence was used, as is common with standard AONs. The extent of non-specific gene silencing also decreased with increasing graft density, suggesting a role for carrier-cell interactions in the non-specific silencing (Fig. 4B). For the most effective treatments using 1% PAO graft copolymers, the silencing effects with bcl-2 sequence were significantly higher than treatments with scrambled AON sequence.

3.6. Biodistribution

As a first evaluation of the disposition of these graft copolymer nanocomplexes *in vivo*, the biodistribution of Cy5.5-AON delivered using DOTAP/AON ('no polymer') and 1%, and 10% PAO-graft copolymer delivery systems was determined in mice with xenograft tumors that were formed using the same A2780 human ovarian cancer cells used for the *in vitro* studies. These conditions were chosen to determine the effects of polymer chemistry, PEO vs. Jeffamine, and grafting density, low (1 mol%) vs. high (10 mol%). DOTAP/ODN/PPAA or 'PPAA' has not been included because, under the formulation conditions for ODN dosing *in vivo*, Cy5.5 ODN precipitation was observed. This condition was not sufficiently stable electrostatically to be included for the biodistribution study. At AON concentrations of 2 µg/mL or lower (that used for physical and *in vitro* studies), all formulations formed stable complexes with DOTAP/AON with negligible free AON in solution [27]. For *in vivo* studies, an AON concentration of 50 µg/mL was determined to be the minimum concentration necessary for the detection of Cy5.5 AON using an IVIS imaging system. At this concentration of DOTAP/AON, the addition of PPAA destabilized the nanocomplex as seen by precipitation of Cy5.5-AON in solution and values greater than 50% free (i.e. unbound AON) in fluorescence quenching studies (data not shown). On the other hand, addition of PPAA-g-PAO stabilized the complex, with minimum free AON in solution, even at high concentrations (data not shown). On this basis, treatments with PPAA were eliminated from *in vivo* studies since sufficient nanoparticle stability and therefore nanoparticle concentrations could not be achieved.

For all conditions, the accumulation of AON 24 hours following i.p. injection was greatest in the kidney, compared to the spleen, liver, lung and tumor (Fig. 5A and B). This likely represents Cy5.5-AON that is released from nanocomplexes throughout the body and subsequently cleared to the kidney by renal filtration, as opposed to direct distribution of the nanocomplexes to the kidney. A greater percentage of Cy5.5-AON was found in all organs at 24 hours when delivered as part of 1% PAO nanocomplexes, suggesting that these particles distributed more extensively and persisted longer in the body compared to DOTAP/AON, where AON was cleared more rapidly.

The treatments with 1 mol% Jeffamine M2070 ('Pg1%J70') and 1 mol% PEO ('Pg1%PEO') showed highest distribution to A2780 xenograft tumors compared to DOTAP/AON ('No polymer') and 10% PAO grafts (Fig. 5C). Although it is known that the accumulation of AON in the tumor is low for untargeted particles compared to targeted particles, the serum-stable nanoparticles with 1% PAO grafts display a significant degree of accumulation in the tumor compared to other formulations tested in this study.

3.7. Bcl-2 gene silencing in tumors

From the biodistribution data, it was determined that the 1% PAO graft copolymer delivery systems were most effective in delivering AON to the tumors, compared to control (DOTAP/AON) and 10 mol% PAO graft copolymer systems. To determine whether AON delivered to tumors with these formulations can reach cells in active form, the ability to induce antisense gene silencing in A2780 xenograft tumors was evaluated for the most promising PAO delivery systems. The intratumoral route was chosen to assess nanoparticle performance independent of nanoparticle stability, which was evaluated through the biodistribution studies. Treatments with the ternary nanoparticle complexes of PPAA-g-1% Jeffamine M 2070 and PPAA-g-1% PEO reduced expression of the bcl-2 gene to 40 and 60%, respectively, at 24 h post-treatment, while the binary DOTAP/AON complex and all of the delivery systems with scrambled AON did not reduce gene expression (Fig. 6). The degree of gene silencing was significant compared to treatments with scrambled sequence (~100% expression of bcl-2 gene), which demonstrated the sequence-specific nature of the antisense gene silencing effect.

4. Discussion

The future success of antisense therapy is highly dependent on the development of efficient and non-toxic carriers. Critical features of a nanoscale drug carrier include extended circulation time in the bloodstream, selective and efficient delivery of cargo to the diseased site, ability to transverse the ECM and other tissue barriers to reach the target cells, and entry into cells to exert a therapeutic effect [53, 54]. We have designed multifunctional copolymers both to improve the biodistribution profile *in vivo* and to achieve cellular association and intracellular AON trafficking. It is key that the carrier properties be balanced between being sufficiently hydrophilic to avoid serum protein attachment and sufficiently lipophilic to achieve cell membrane association. To actuate this design, we synthesized grafts of poly (alkylene oxide) (PAO) onto pH-sensitive membrane sensitive polymers. By developing and studying a panel of graft copolymers with varying graft polymer type and graft density, we established the influence of polymer HLB on membrane association, pH-sensitive membrane lysis, and *in vitro* gene silencing. Further, an effective DOTAP/AON/polymer carrier system with increased accumulation of AON in the solid tumor and increased gene silencing of the bcl-2 gene was found.

Limited previous studies have investigated the effect of carrier hydrophobicity on overall gene transfection (or gene silencing). Here we explore in greater depth the effect of HLB by assessing its influence not only on gene silencing but also on the important steps involved in the process of nucleic acid delivery including liposome membrane penetration and the ability to penetrate membranes at endosomal pH. In this work, ungrafted PPAA, which is relatively lipophilic (low HLB), displayed the greatest membrane/cell association, hemolysis and gene silencing effects (in the form of DOTAP/AON/PPAA complexes) in serum-free media. PPAA appears to possess an excellent combination of lipophilicity and pH-dependent protonatable groups to enter cells and escape endolysosomal compartments. However, we have observed that DOTAP/AON/PPAA is less effective in serum containing media, to an extent that depends on cell type. Therefore, it is necessary to graft moieties on

to the polymer to reduce serum interactions. An overriding hypothesis of our work is that the overall performance of the delivery system can be optimized by varying the PEO/PPO density and ratio in grafted PAO polymers.

The hemolytic activity and liposome membrane penetration ability of the graft copolymers are driven by their physicochemical interactions, including inter-chain hydrogen bonding between PPAA backbones and steric interactions, along with solvation effects and van der Waals forces of the grafted PAO chains. A complete theoretical description of these complex and inter-related interactions is beyond the scope of this study, but a general hypothesis is that the greater the overall self-associative forces of the polymer or graft copolymer, the more likely it was to form a disruptive zone within the cell membrane and thereby increase permeability. In this light, the strong influence of HLB on membrane association observed in the calcein leakage assay is not surprising (Fig. 7A). Furthermore, it is known that the hydrogen bonding interactions of poly(alkylacrylic acids) are pH-dependent [55–57]. The pH-induced conformational changes in the PPAA chemistry explain its membrane penetrative activity at acidic pH. The lack of correlation between HLB and RBC lysis (at pH 6.0) likely reflects these changes in polymer structure (Fig. 7B). The greater the graft density of PPAA, the less would be the hydrogen-bonding attraction between backbone PPAA's primarily due to steric hindrance by the grafted chains but secondarily because there are fewer carboxylic acid groups in the backbone. This helps explain the behavior of the 5 and 10% grafted polymers, where membrane association is abrogated in both the calcein leakage and hemolysis assays. In the case of the most hydrophobic Jeffamine M-2005, self-association was increased by enhanced lipophilic interactions of the propylene oxide groups in the grafted Jeffamine chains. The highest hemolytic activity was observed in the case of this polymer with greatest HLB but also the highest degree of cytotoxicity (Figs. 1, 3).

Although the lipophilicity of PPAA favors membrane association, in the presence of serum, nanocomplexes formed with PPAA exhibited greatly reduced gene silencing as compared to those incorporating 1% PAO graft copolymers (Fig. 7C). In our previous work, we showed that PPAA nanocomplexes were able to achieve endosomal escape when they entered cells, but they exhibited low cell association in serum-containing media. While increasing the graft density of hydrophilic polymer onto PPAA decreased membrane association and gene silencing effect, some level of grafting was necessary to achieve maximum gene silencing in the presence of serum (Fig. 7C). The 1 mol% PAO grafted copolymer contained a sufficient degree of hydrophobicity to associate favorably with membranes. While it is possible that the corresponding values of HLB (e.g., 0.07 for 1 mol% Jeffamine graft) represent an optimum, it is more likely that the greater effectiveness of low HLB polymers is subject to the constraint that there must be sufficient polymer grafted to prevent aggregation or degradation in the presence of serum. Taken together, this set of correlations (Fig. 7A–C) show that effective gene silencing by DOTAP/AON/PAO complexes in serum requires a delicate interplay of serum stabilization, cell membrane association and pH-dependent endosomal escape. Consideration of any one of these factors in isolation is insufficient to define the most effective polymer for gene silencing.

Often, the most effective delivery systems in cell culture are not well suited for *in vivo* use. Nonetheless, because our delivery system was designed *a priori* with multiple functionalities for serum stability and intracellular delivery, our hypothesis was that the promising *in vitro* results of the 1 mol% PAO-copolymer delivery system would translate *in vivo*. Indeed, the liposomal formulation DOTAP/AON shows negligible Cy5.5-AON accumulation in the tumor, with a majority in the liver and spleen, suggestive of RES uptake. On the other hand, incorporation of PPAA with 1 mol% grafting of Jeffamine M-2070 or PEO led to significantly higher accumulation of labeled AON in the tumor compared to DOTAP/AON and the copolymer formulation with 10 mol% grafting (Fig. 5). The majority of AON accumulation using the 1 mol% graft copolymer treatments was seen in the kidney, followed by the spleen and liver, and to a small degree in the lungs. We reason that over the course of 24 hours in the body, the equilibrium between bound and free AON will result in gradual release throughout the body, increasing free AON of low molecular weight, which then accumulates in the kidney by the small size/MW cutoff. In the case of the other carriers, clearance occurs more rapidly, such that by 24 hours most of the AON has already been eliminated from the body. The greatest AON accumulation in the tumor was observed in the complexes containing the 1 mol% PAO-delivery systems. Furthermore, the results of the intratumoral gene silencing validate the successful delivery of AON using the graft copolymer delivery system in an *in vivo* tumor model.

5. Conclusions

These studies show a definite positive relationship between lipophilicity (or inversely between HLB) of PPAA-g-PAO graft copolymers and membrane association. This relationship appears to be critical to the finding that lower HLB values for these copolymers lead to greater gene silencing in serum containing media when used in conjunction with otherwise ineffective DOTAP/AON, with the proviso that ungrafted PPAA is ineffective in serum, presumably due to serum-mediated aggregation or degradation. Notably, we have found that the 1 mol% Jeffamine and PEO graft copolymer delivery systems proved to be most effective for antisense delivery not only in cell culture but also *in vivo*. Specifically, these polymers have shown particle stability, increased tumor accumulation upon systemic administration and bcl-2 target gene silencing upon intra-tumoral administration. As such, the DOTAP/PPAA-g-PAO system shows considerable promise for delivery of AONs for gene silencing *in vivo*.

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Figure 1.

Effect of grafting on pH-dependent hemolysis of PPAA, normalized to RBCs in distilled water (100%). PPAA is denoted as 'P', PPAA-g-1%Jeffamine M 2005 as 'Pg1%J05', PPAA-g-1%Jeffamine M 2070 as 'Pg1%J70', PPAA-g-1%PEO as 'Pg1%PEO'. The 5 and 10 mol% graft copolymers showed negligible lysis (not shown on graph). Data represent mean $\pm$ s.d. ($n=3$); asterisk (*) denotes significance at a value of $p < 0.05$.

Figure 2. Membrane activity assayed by release of calcein dye from DPPC liposomes upon incubation with A) Jeffamine graft copolymers and B) PEO graft copolymers. A value of 100% calcein release was obtained upon incubation of DPPC liposomes with Triton X-100 surfactant. Refer to all legend descriptions in Fig. 1. Note: PPAA-g-1%Jeffamine M-2005 showed 100% calcein release throughout the concentrations tested. Data represent mean $\pm$ s.d. ($n=3$); asterisk (*) denotes significance at a value of $p < 0.05$.

Figure 3. Nanocomplexes formed with highly lipophilic graft copolymers compromise A2780 cell metabolic activity. Cells were treated with DOTAP/Polymer complexes delivering sequence-specific antisense for 4 h under 10% FBS-containing media conditions. Cell metabolic activity was measured 48 h from the start of treatment using the MTT assay. Refer to Figure 1 caption for polymer abbreviations. Condition 'Pg1%J05' is statistically different ($p < 0.05$) from all other samples (except PPAA 'P') ($n=3$, errors bars represent s.d.).

Figure 4.

Nanocomplexes containing graft copolymers mediate effective and sequence-specific knockdown. Gene expression of bcl-2 in A2780 cells upon treatment with various complexes containing A) bcl -2 antisense and B) scrambled antisense for 4 h under 10% FBS-containing media conditions. Bcl -2 gene expression was measured 24 h from the start of treatment. The net charge ratio for DOTAP/ODN ('No polymer') is 4.7 and DOTAP/ODN with various polymers is 1.0. Refer to Figure 1 captions for polymer abbreviations. Data represent mean $\pm$ s.d. ($n=3$); asterisk (*) denotes significance at a value of $p < 0.05$.

Figure 5.

Biodistribution of Cy5.5 to the kidney (B) and tumor (C) is enhanced for nanocomplexes formed with 1%PAO graft copolymers, compared to 'No polymer' (DOTAP carrier with no polymer). A) IVIS images of excised organs from 3 representative mice injected with Cy5.5-AON complexed with the indicated delivery systems. Scale bars indicate range of Cy5.5 signal intensity. Quantification of accumulation of Cy5.5-labeled ODN in organs of B) kidney, spleen, liver and lung organs, and C) tumor, 24 h post-treatment of mice with i.p injected nanocomplexes. Fluorescence from kidney, liver, lung and spleen were normalized to mass (g) and tumor to volume (cm³). Note: accumulation in heart was negligible (quantitative data not shown). Refer to polymer abbreviation in captions of previous figures. Data represent mean $\pm$ s.e.m. ($n = 4-6$); asterisk (*) denotes significance at a value of $p < 0.05$.

Figure 6.

Gene silencing in a tumor is achieved for nanocomplexes formed with 1% PAO graft copolymers. Bcl-2 gene expression in A2780 xenograft tumors was determined 24 h following -intratumoral injection with indicated nanoparticle formulations. Formulations include bcl-2 specific antisense sequence except where indicated scrambled 'sc' sequences. Data represent mean $\pm$ s.d. ($n = 4-5$); asterisk (*) denotes significance at a value of $p < 0.05$.

Figure 7.

HLB is related to membrane association and gene silencing. The HLB factor is a sum of the hydrophilic and lipophilic groups present in one mole of the polymer. Relationship is shown of polymer rHLB (relative hydrophilic lipophilic balance) to (A) Calcein dye release from DPPC liposomes, (B) RBC lysis at pH of 6.0, and (C) % bcl-2 gene expression in A2780 cells.

PEO

Jeffamine M-2070

Jeffamine M-2005

Scheme 1.

Synthesis of PPAA graft copolymers with PEO, Jeffamine M-2070 and Jeffamine M-2005 graft chemistries with 1, 5 and 10 mol % graft density.

Table 1

Characteristics of poly(alkylene oxide) graft copolymers

Graft copolymer/MW	Designation	Target graft density (mol%)	NMR graft density (mol%)	Solubility	HLB
Jeffamine M-2070/2kDa	'Pg1%J70'	1	3	Aqueous	0.07
Jeffamine M-2070/2kDa	'Pg5%J70'	5	8	Aqueous	0.19
Jeffamine M-2070/2kDa	'Pg10%J70'	10	11	Aqueous	0.26
Jeffamine M-2005/2kDa	'Pg1%J05'	1	2	Aqueous	-0.02
Jeffamine M-2005/2kDa	---	5	--	Organic	-0.06
PEO/2kDa	'Pg1%PEO'	1	3	Aqueous	0.13
PEO/2kDa	'Pg1%PEO'	5	12	Aqueous	0.52
PEO/2kDa	'Pg1%PEO'	10	23	Aqueous	1.00

* Note: Molecular Weight of parent polymer, poly (propylacrylic acid) or 'P' is 200 kDa