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Effects of Base Polymer Hydrophobicity and End Group Modification on Polymeric Gene Delivery

Joel C. Sunshine, Marib I. Akanda, David Li, Kristen L. Kozielski, and Jordan J. Green*

Department of Biomedical Engineering, The Johns Hopkins University School of Medicine, 400 N. Broadway / Smith Building Room 5017, Baltimore, MD 21231

Abstract

A new 320 member polymer library of end-modified poly(beta amino) esters was synthesized. This library was chosen such that small differences to the structures of component backbone, side-chain, and end-group monomers could be systematically and simultaneously evaluated. The *in vitro* transfection efficacy and cytotoxicity of DNA nanoparticles formed from this library was assessed. This library approach enabled us not only to synthesize and test a large variety of structures rapidly, but provided us with a robust dataset to analyze for the effect of small structural permutations to polymer chain structure. Small changes to the side chains, backbones, and end groups within this polymer library produced dramatic results, with transfection efficacy of CMV-Luc varying over 4-orders in a 96-well plate format. Increasing hydrophobicity of the base polymer backbone and side chain tended to increase transfection efficacy, but the most hydrophobic side chains and backbones showed the least requirement for a hydrophobic pair. Optimal PBAE formulations were superior to commercially available non-viral alternatives FuGENE® HD and Lipofectamine™ 2000, enabling \sim 3-fold increased luminescence (2.2×10^6 RLU/well vs 8.1×10^5 RLU/well) and 2-fold increased transfection percentage (76.7% vs 42.9%) as measured by flow cytometry with comparable or reduced toxicity.

Keywords

Non-viral; Gene delivery; Biodegradable polymer; Combinatorial library; Hydrophobicity

Introduction

Gene therapy holds out the promise of specific therapy designed to target the root causes of a plethora of diseases, ranging from single gene diseases such as sickle cell anemia and hemophilia, to diseases with a genetic basis such as cancer, diabetes, and heart disease. Since viruses have long evolved to be exceptionally efficient at getting their genetic information into cells, scientists and clinicians initially took advantage of this to develop viral vector-based gene therapeutics. Unfortunately, the field had significant setbacks when tragedies occurred in clinical trials, including deaths due to excessive immune responses to the viruses, as well as subsequent cancer generation due to insertional mutagenesis.^{1–3} The major effort in this field remains in the viral arena – 75% of clinical trials for gene therapy use viral vectors.⁴ Due to the potential drawbacks of viral vectors, including their

green@jhu.edu.

Supporting Information Available

Supplementary tables, as cited in the text, are available which show the data in heat-map versions with all of the raw data shown. In addition, all other supplementary figures are also available in the supplementary information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

immunogenicity, potential for insertional mutagenesis, small cargo capacity, and difficulty involved in large scale production, a wide variety of non-viral vectors have been investigated for their nucleic acid delivery efficacy.^{5, 6} Among the polymers investigated, poly(beta-amino) esters have particularly shown promise as gene delivery vectors,^{7–11} with some formulations rivaling adenoviral transfection efficacy in hard to transfect human cell lines.¹² Promising recent applications of these polymers include their use in cancer therapy,^{13–15} as electrostatic coatings on gold nanoparticles for efficient delivery of siRNA,¹⁶ as biodegradable hydrogels,^{17, 18} and as pH-responsive components of polymeric micelles for drug release.¹⁹

Poly(beta-amino) esters (PBAE) are synthesized by simple Michael addition of diacrylates to primary (or bis-secondary) amines. Previous studies have noted that amine-terminated versions of the polymers were far superior to corresponding diacrylate-terminated versions,⁸ and thus have investigated whether modification of the ends of the polymers would have a dramatic effect on transfection efficacy.²⁰ Modification of the ends of the polymer has been shown to be important not only with regard to improving the efficacy of a particular polymer backbone, but the particular end group which is optimal appears to be a function of the cell type investigated.²¹ There is a relationship between which polymers transfet well in 2D as compared to 3D culture methods, but the relationship is not perfect and some formulations can be better at transfecting cells in 2D monolayer or 3D culture.²² Some convergence of optimal structure has been previously reported, including that small particle sizes have been associated with improved transfection, and that optimal polymers tend to contain amino alcohol side chains.²³

One additional advantage of this library approach to vector design is that it can provide the means to productively analyze structure function relationships across a wide or narrow window of potential structures. However, previous studies have not looked at the systematic modification of all three structural and chemical elements that compose the polymer: backbone, side chain, and end-group. Here, we do this and synthesize a new library of 320 end-modified PBAEs that differ by single carbon changes to the monomers that compose the backbone and side chains and small functional changes to the end-group. In this way we can analyze the contributions of spacer length, side chain length, and overall hydrophobicity / hydrophilicity of the end-modified polymer to the resulting transfection efficacy.

We characterize the transfection efficacy and toxicity of the nanoparticles that these polymers form with DNA through self-assembly. The best performing polymer formulations show superior transfection efficacy to commercially available alternatives with better toxicity profiles, and the data reveal insight into structure function relationships within this polymer library.

Experimental

Materials

All reagents and solvents were obtained from commercial suppliers and used as received. Monomers were purchased from Acros Organics [1-(3-aminopropyl)-4-methylpiperazine (E8)], Alfa Aesar [3-amino-1-propanol (S3), 4-amino-1-butanol (S4), 5-amino-1-pentanol (S5), 1,4-butanediol diacrylate (B4), 1,6-hexanediol diacrylate (B6), 1-(3-aminopropyl)-4-methylpiperazine (E7)], Fluka [2-(3-aminopropylamino)ethanol (E6)], Monomer-Polymer and Dajac Labs [1,3-propanediol diacrylate (B3), 1,5-pentanediol diacrylate (B5)], Sigma Aldrich [3-aminopropane-1,2-diol (S3o), 1,3-butanediol diacrylate (B3m), 2-(benzoyloxymethyl)-2-ethylpropane-1,3-diyl diacrylate (BL1), ethoxylated bisphenol A diacrylate (BL2), glycerol 1,3-diglycerolate diacrylate (BH1), 1,3-diaminopropane (E1), 4-aminophenyl disulfide (E9), cystamine dihydrochloride (E10), 2-(1H-imidazol-4-

yl)ethanamine (E12)], and TCI America [1,3-diaminopentane (E3), 2-methyl-1,5-diaminopentane (E4), (PEO)₄-bis-amine (E5)]. Plasmids (CMV-Luc and CMV-eGFP) were amplified by Aldevron (Fargo, ND) and used as received. FuGENE[®] HD, LipofectamineTM 2000, and Opti-MEM I were purchased from Invitrogen and used according to manufacturer instructions. CellTiter 96[®] AQueous One MTS assay and the BrightGloTM assay system were purchased from Promega, and were used according to manufacturer's instructions. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen and supplemented with 10% FBS and 1% penicillin/streptomycin.

Methods

Polymer Library Synthesis—The library of PBAEs was synthesized by adding primary amines (S) to diacrylate (B) compounds (1.2:1 molar ratio of diacrylate:amine, 5 g total reaction mass) without solvent, stirring at 1000 rpm at 90 °C for 24 hours. In a second step, the base polymers were dissolved in anhydrous DMSO (Sigma Aldrich) at 167 mg/ml. 480 microliters of base polymer at 167 mg/ml (80 mg) was then mixed with 320 microliters of a 0.5M solution of one end-capping amine (E), and allowed to react, while vortexing at 1000 rpm (VWR shaker) at room temperature for 24 hours. Eight diacrylate bases, 4 amino alcohol side chains, and 10 primary-amine containing end-groups were used to synthesize 320 total polymers.

Gel Permeation Chromatography—Organic phase GPC was performed using 94% THF/5% DMSO/1% piperidine (v/v) as the eluent at a flow rate of 1.0 ml/minute in a Waters GPC system equipped with a Waters 717plus autosampler (Waters Corporation, Milford, MA). Three Waters Styragel columns (HR1, HR3, and HR4) were used in series, and the detector (Waters 2414 refractive index detector) and columns were maintained at 40 °C throughout the runs. The molecular weights of the polymers are reported relative to monodisperse polystyrene standards (Shodex, Japan). 100 µl of each sample prepared at 5 mg/ml was injected, and each sample was given 40 minutes to elute off of the column.

NMR—All 32 base polymers and at least one representative end-modified polymer for each of the 10 end-modification reactions were characterized on a Bruker spectrometer by ¹H-NMR spectroscopy (400 MHz, d₆-DMSO); for the complete spectra of all the polymers, see supplementary information. As an example, for base polymer B3-S5: 1.15–1.25 (2H, quint, NCH₂CH₂CH₂CH₂OH), 1.25–1.35 (2H, quint, NCH₂CH₂CH₂CH₂CH₂OH), 1.35–1.45 (2H, quint, NCH₂CH₂CH₂CH₂OH), 1.85–1.95 (2H, quint, CH₂CH₂NCH₂CH₂(COO)CH₂CH₂CH₂(COO)), 2.3–2.4 (6H, t, CH₂CH₂NCH₂CH₂(COO)CH₂CH₂CH₂(COO)) and t, NCH₂CH₂CH₂CH₂CH₂OH), 2.6–2.7 (4H, t, CH₂CH₂NCH₂CH₂(COO)CH₂CH₂CH₂(COO)), 3.3–3.4 (2H, obs, NCH₂CH₂CH₂CH₂OH), 4.0–4.1 (4H, t, CH₂CH₂NCH₂CH₂(COO)CH₂CH₂CH₂(COO)), 4.1–4.2 (t, CH₂(COO)CH=CH₂), 4.25–4.35 (br, NCH₂CH₂CH₂CH₂OH), 5.9–6 (d, COOCH=CH₂), 6.1–6.2 (dd, COOCH=CH₂), 6.3–6.4 (d, COOCH=CH₂). For the end-modified polymers, end-modification was verified by the disappearance of the peaks at 5.9–6, 6.1–6.2, and 6.3–6.4 ppm which correspond to the acrylate protons. However, the E9 reactions with base polymers left residual acrylate protons after reaction, indicating incomplete reaction and presence of the acrylate-terminated base polymers. All the other end-modifying amines resulted in complete elimination of acrylate peaks, corresponding to a complete reaction.

Amide formation (peaks at 7.9 ppm corresponding to CO-NH and 3.0–3.2 corresponding to CONHCH₂) was noted in a subset of the end-modified polymers, but not in any of the base polymers. Amide formation (quantified by the ratio of peaks corresponding to CONHCH₂ and COOCH₂) was highest with polymers modified with E1, E3, E4 (two primary amines),

was moderate with polymers modified with E6 (one primary, one secondary amine), and was minimal in end modified polymers containing E5, E7, E8, E10, and E12 (Table S1). Increasing amide formation also resulted in decreased molecular weight of the end-modified polymers (Figure S1) with the same pattern (Figure S2, linear regression $R^2 = 0.5637$, $p < 0.0001$), indicating that amide formation was the direct cause of the decrease in molecular weight seen with the end-capping step. These trends, however, do not appear to have any impact on the transfection efficacy of the resulting end-modified polymer (Figure S3, linear regression $R^2 = 0.0605$, $p > 0.05$; not significant), indicating that small extent of amide bond formation and resulting decrease in molecular weight do not significantly impact the transfection efficacy of the end-modified polymers.

Polymer Solubility—Solubility was measured for a subset of polymers in the buffer used to dissolve the polymers and form the nanoparticles (25 mM sodium acetate (NaAc) in water) through a plate-reader absorbance assay. Ten microliters of 100 mg/ml polymer solution in DMSO was added to 40 microliters of 25 mM NaAc, forming a milky mixture. Absorbance of each well at 620 nm was measured with a plate reader (BioTek Synergy 2). Sequentially, each well was diluted by addition of another 10 microliters of 25 mM NaAc buffer, was mixed by pipetting up and down 5 times, and the resulting well was re-measured with the plate reader. Complete solubility was determined by comparing the absorbance at 620 nm for each well with a reference well containing the same amount of DMSO and 25 mM NaAc, and the result was also confirmed by eye.

Luciferase Transfection and Viability Testing—COS-7 cells were seeded at 15,000 cells/well (50,000 cells/cm²) into 96-well plates in complete DMEM and allowed to adhere overnight. Polymers were then aliquoted into 96-well U-bottom plates and dissolved in 25 mM sodium acetate buffer (pH 5.2). Separately, CMV-Luc DNA (Elim Biopharm) was diluted and aliquoted out. Diluted polymer was added to CMV-Luc DNA using a multichannel pipette and mixed vigorously by pipetting up and down. Nanoparticles were given 10 minutes to complex, and then were added to cells (20 microliters of nanoparticles added to 100 microliters of fresh complete DMEM). Final particle composition for all polymers was 600 nanograms of CMV-Luc DNA and 36 micrograms of polymer (60 wt/wt polymer:DNA ratio). As positive controls, LipofectamineTM 2000 (Invitrogen) and FuGENE[®] HD were prepared in Optimem I (Invitrogen) according to manufacturer's instructions and added to cells in the concentrations described in the text. After four hours of incubation, the media (and remaining particles) were removed by pipetting, and the media was replaced with fresh warmed DMEM. Twenty four hours after transfection, metabolic activity was assessed by the CellTiter 96[®] AQ_{ueous} One MTS assay (Promega) and was normalized to untreated control wells. Briefly, 20 microliters of assay reagent was added to cells. Cells were placed back in the incubator for 1 hour, and then absorbance of each well at 590 nm was measured with a plate reader (BioTek Synergy 2). Plates were washed with 1× PBS and fresh media was added to each plate. 48 hours after transfection, luminescence was measured on a plate reader using the BrightGloTM luciferase assay system. Briefly, 100 microliters of room-temperature assay reagent was added to 100 microliters of media on cells. The plate was swirled for precisely 2 minutes, and then the luminescence was measured.

GFP Transfection and Flow Cytometry—Cell plating, particle formulation, and transfection protocol for the GFP transfection was the same as above, except using EGFP-N1 DNA (Clontech) and particles were formulated at 30, 60, and 90 polymer:DNA wt:wt ratios instead of only 60 wt. 48 hours post transfection, the cells were washed and trypsinized with 30 microliters of 0.25% trypsin-EDTA. 170 microliters of FACS buffer (1× PBS, 2% FBS, 0.5% propidium iodide) was added to cells and the 200 microliters was

transferred to Starstedt 96-well round-bottom plates. The plates were centrifuged for 5 minute at 1000 rpm, removed from the centrifuge, and 170 microliters was removed from on top of the cell pellet. The cell pellet was resuspended in the residual 30 microliters and placed on an Intellicyt high-throughput loader and reader attached to an Accuri C6 flow cytometer. Each well was run dry, and in between each well we included a 1-second PBS wash to minimize well to well contamination. The Hypercyt software was used to discriminate between wells and FlowJo was used for FACS analysis.

Results and Discussion

Characterization of the Polymer Library—The library of PBAEs was synthesized by adding primary amines to diacrylate compounds (1.2:1 molar ratio of diacrylate:amine) at 90 °C for 24h (Scheme 1A). These specific monomers were chosen so that single carbon changes to the backbone monomers and to the side chain monomers could be evaluated in the synthetic polymers. In a second step, the base polymers were end-capped by end-capping amines (at 10-fold molar excess of amine to diacrylate termini) at room temperature for 24 hours (Scheme 1B). These end groups were chosen so that the presence of small molecule functional groups could be evaluated and compared across base polymers with differential structure. Eight diacrylate bases, 4 amino alcohol side chains, and 10 primary-amine containing end-groups were used to synthesize 320 total polymers (Scheme 1C). In order to more closely match the underlying structure to the naming convention used, we have chosen a separate naming convention from previous studies. Here, the number after “B” (for “base”) corresponds to the number of carbons between acrylate groups in the diacrylate, so B3 means than there are 3 CH₂ units between acrylate groups in the diacrylate base. The number after “S” (for “side chain”) corresponds to the number of carbons between the amine group and the hydroxyl group in the side-chain. Previous top-performing base polymers termed “C32”, “C28”²⁴ correspond to B4-S5 and B4-S4 respectively. The “E” (for “end group”) refers to which end-modifying amine was chosen; they are organized into structurally similar groups but the numbers are simply sequential. The modifier “m” refers to an added methyl group (so B3m has an added methyl group compared to B3) and the modifier “o” to an added hydroxyl group (so S3o has an added hydroxyl group compared to S3). Every base polymer was characterized with respect to its base polymer molecular weight. Weight-averaged molecular weights of the polymers in the library ranged from 2,000 to 48,000, and number-averaged molecular weights ranged from 1,500 to 12,000 (Figure 1, Table S2). While many of the base polymers (8/32) had a weight-averaged molecular weight (Mw) of approximately 10,000 Da (+/- 2,000 Da), some polymers had an especially high Mw (40–50,000 Da, such as B4-S4, B6-S4, and BL2-S3o), and others had an especially low MW (under 3,000, such as BL1-S3 and BH1-S4). The viscosity of the starting monomers appears to have a significant effect on the molecular weight obtained using this synthesis protocol. S3o is relatively more viscous than S3, S4 or S5; polymers synthesized with S3o all had Mw of less than 9,000 Da, except for BL2-S3o. S4 containing polymers with simple hydrocarbon backbones (B3, B3m, B4, B5, B6) all had Mw of greater than 10,000. BL1, BL2, and BH1 all needed to be solvated in order to be effectively mixed with the amino alcohol side chains, and the resulting polymers in general were all smaller than the Mw obtained from the neat synthesis protocol.

Effect of Polymer:DNA ratio—To form nanoparticles, PBAEs were dissolved in 25mM NaAc (pH 5.2) to generate positive charge on the amines and complexed with CMV-Luc DNA at varying polymer:DNA weight ratio (wt/wt). Before screening the entire library at a particular polymer:DNA ratio, we looked at the effect of formulation ratio on polymer transfection efficacy. To do this, we tested a subset of 21 representative polymers for gene delivery at a wide range of wt/wts: 30, 60, 80, 100, 125, and 150 (Figure 2, Table S3). Maximal luminescence was achieved at 60 wt/wt for most polymers tested. However,

interestingly for B3-S5 end-modified polymers, maximal luminescence intensity was not achieved until a high 125–150 wt/wt ratio. Since maximal luminescence in general was achieved at 60 wt/wt ratio, we used this wt/wt ratio when subsequently screening the entire library of 320 structures. By testing all polymers at the same weight ratio, the influence of structure can directly be evaluated without the formulation ratio producing possible confounding effects.

Effect of Base Polymer Composition—To evaluate the effect of base-composition of the polymers on transfection efficacy, polymers were allowed to spontaneously form nanoparticles at a fixed weight ratio (60 wt/wt) and then added to COS-7 cells as and examined for transfection efficacy by total well luminescence from BrightGlo (Figure 3, Table S4). To quantify cytotoxicity/cell viability, separate experiments were also conducted in parallel with the same nanoparticles and following the same transfection procedure. Twenty-four hours after incubation, metabolic activity was assessed by the CellTiter 96[®] AQueous One MTS assay and was normalized to untreated control wells (Figure 4, Table S5).

Previous studies have shown that polymer molecular weight may play a role in transfection efficacy, with increasing molecular weight corresponding to increasing transfection efficacy.²³ At least within this library of polymers, this effect appears to be muted (Figure 5), as there was no correlation between increasing molecular weight of the base polymer and increasing average transfection efficacy for all end-modified polymers from the same base polymer (linear regression, $R^2 = 0.003$, $p = 0.7833$),.

Polymers containing simple hydrocarbon backbones were more effective than polymers containing bulkier hydrophobic or hydrophilic backbones. In particular, polymers containing the bulkier hydrophobic backbones (BL1 and BL2) were toxic to the cells at the tested wt/wt and DNA dose, while polymers containing the hydrophilic backbone (BH1) were relatively non-toxic but did not promote transfection.

Single carbon changes to the base polymers in the polymer library produced dramatic results for transfection efficacy. Interplay was found between the relative hydrophobicity and hydrophilicity of the diacrylate and amino alcohols used to synthesize the base polymers. To evaluate the differences seen by this library approach statistically, we converted the luciferase luminescence data for the end-modified polymers containing B3, B3m, B4, B5, and B6 diacrylates and S3o, S3, S4, and S5 side chains to log-scale and performed analysis of variance (GraphPad Prism).

Other studies have looked at modifying cationic polymers with hydrophobic moieties to increase transfection efficacy. Hydrophobic modification of polycations has been shown to be beneficial for gene delivery for a variety of reasons. Increased hydrophobicity may enhance polymer-DNA binding by providing for physical encapsulation, in addition to charge-charge interactions.²⁵ Additionally, a general mechanism for uptake of non-viral gene delivery particles results from adsorptive endocytosis; enhancing the hydrophobicity of the polymer could result in increased interaction with the cell membrane and promote this process.²⁵ Hydrophobic modifications have been also used to improve gene dissociation from the polycation, by decreasing the electrostatic interactions between the cationic polymer and DNA^{26, 27}, and have been shown to improve the performance of the gene-carrier in the presence of serum.²⁸

A few excellent recent examples of studies looking at hydrophobic modification of a polycation base polymer show that with hydrophobic modifications, usually some modification improves the product, but too much hydrophobicity can decrease efficacy. N-

alkylation of linear polyethyleneimine (PEI) with varying alkyl chain lengths (1, 2, 3, 4, and 8-carbon chains) at 11% of the backbone amines produced dramatic results. Gene delivery to the lung in a mouse model increased 8-, 26-, and 7-fold when modified by 1, 2, or 3 carbons, decreased moderately when modified by 4 carbons, and decreased substantially (200-fold) when modified by 8-carbon chains, as compared to 22 kDa linear PEI.²⁹ In addition, 11% alkylation produced the most transfection boost with N-ethyl-PEI, as compared to derivatization of 5%, 14%, or 20% of the backbone amines.²⁹ In other work, poly(amidoamine) (PAMAM) dendrimers were functionalized with 4–8 alkyl chains (12–16 carbons in length).³⁰ Cellular uptake was improved by both increasing the amount of functionalization and increasing length of alkyl-chain modification (as these chains act as a lipid coating for the PAMAM dendrimer), whereas transfection efficacy was optimal with the shortest chain length (12-carbons).³⁰

Modification of chitosan, a naturally occurring polycation commonly used as a non-viral gene delivery reagent, with hydrophilic and hydrophobic chains resulted in dramatic effects on the gene delivery properties of this modified polymer. Both modifications enhanced plasmid release and reduced non-specific adsorption, but only modification with the hydrophobic PMLA enhanced cell adsorption and cellular uptake.³¹

In comparison to these studies, our study examines how directly modulating the hydrophilic / hydrophobic character of an end-modified cationic polymer (rather than adding hydrophobic moieties to an existing cationic polymer) might modulate transfection efficacy. Presumably, some of the advantages that would be granted to cationic polymers by modifying them with hydrophobic moieties might be conferred to a polymer which at its core was modified to increase its hydrophobicity. In particular, increasing hydrophobic character of the core polymer might increase the ability of that polymer to physically encapsulate the DNA cargo and may also promote adsorptive endocytosis, as in many of the hydrophobic-modified polymer cases.²⁵

To determine how changing the hydrophobicity of the diacrylate base and the amino alcohol side chain affects the performances of all polymers in the library, we first averaged the log-scale luminescence data for all end-modified polymers with the same base polymer together so that they represent one composite value for each particular base polymer (Figure 6). Interestingly, increasing hydrophobicity of either the diacrylate base or the amino alcohol side chain resulted, in general, in increased transfection efficacy. This trend is shown in the cases of end-capped polymers with B3, B3m, B5, and B6 as backbones and S3o and S3 as side-chains. In the special case of B4-based polymers, only polymer B4-S4 is less effective than otherwise would be predicted. In the case of S4-based polymers, there is biphasic dependence on hydrophobicity with B4-S4 having minimal activity and B3-S4 and B6-S4 having comparable effectiveness. In the case of S5-based polymers, efficacy is high and equivalent across all backbone types.

Closer inspection reveals that there may be some interplay between backbone and side-chain hydrophobicity. Examining the results with respect to increasing amino alcohol side chain hydrophobicity (Figure 6), it is clear that the only polymers which included the most hydrophilic side chain (S3o) to achieve significant transfection used the most hydrophobic B6-S3o backbone. For polymers with the S3 side chains, the most effective base polymers incorporated the two most hydrophobic diacrylates (B5, B6). Among the polymers with the most hydrophobic side chain (S5), the most effective end-modified polymers were largely equivalent, although B4-S5 and B5-S5, with intermediate hydrophobicity, were slightly more effective with particular end-groups. S4 has intermediate hydrophobicity and S4-based polymers were shown to have biphasic dependence on backbone hydrophobicity, with the most hydrophilic (B3) or hydrophobic (B6) backbones being optimal compared to the

intermediate hydrophobicity backbones (B3m, B4, B5). Overall, there was excellent transfection achieved with all five of these base diacrylates tested. These findings suggest that with increasing side chain hydrophobicity, the requirement for a hydrophobic base diacrylate decreases and also suggests that if we were to test even more hydrophobic side chains that we might notice that incorporating diacrylates with reduced hydrophobicity might be optimal.

For all base diacrylates, the optimal side chain in general was the S5 side chain. However, with increasing base diacrylate hydrophobicity, there is decreasing preference for the most hydrophobic side chain (Figure 6). For the least hydrophobic (B3) backbone, there is nearly an order of magnitude increase in transfection efficacy between each increasingly hydrophobic side chain (from 2.94 for B3-S3o to 3.64 for B3-S3 to 4.72 for B3-S4 to 5.55 for B3-S5 in logscale units). For the most hydrophobic (B6) backbone, there is less than an order of magnitude difference in transfection efficacy between the extremes (from 4.49 for B3-S3o to 4.91 for B6-S3 to 4.80 for B6-S4 to 5.40 for B6-S5). This suggests that if we were to test even more hydrophobic base diacrylates, that it is likely that increasing the hydrophobicity of the side chain (by increasing carbon length) may not enhance the gene delivery properties of the resulting end-modified polymer.

To evaluate the relative importance of the base diacrylate and the side chain amino alcohol on the transfection efficacy displayed by the polymer library statistically, we calculated a 2-way ANOVA with our data. In the 2-way ANOVA, the side chains accounted for the largest share of the variance seen (45%, $p < 0.0001$), and the diacrylate used accounted for a smaller, but still highly statistically significant portion of the variance (8.5%, $p < 0.0001$). The interaction between the two groups accounted for an additional 9% of the variance ($p < 0.0001$). This demonstrates that side chain hydrophobicity produced even more dramatic results than increasing base diacrylate hydrophobicity (although both effects are significant). A potential explanation for this discrepancy is that increasing “B” (base diacrylate) hydrophobicity increases spacing between charged nitrogens in the backbone but increasing “S” (side chain) hydrophobicity does not. Thus, increasing the hydrophobicity of the side chains may yield the advantages associated with increased hydrophobicity without interfering with charge spacing, but increasing the hydrophobicity of the base diacrylate both increases the hydrophobicity and increases the spacing between nitrogens. Also of note, increasing the hydrophobicity of either the backbone or the side chain while holding the same DNA to polymer wt/wt ratio constant effectively decreases the nitrogen to phosphate ratio at the same time, which may affect particle formation and DNA release.

Another way to examine bulk hydrophilicity / hydrophobicity of the polymers is to examine the solubility trends of polymers in the library. We took a subset of polymers which were end-modified with E7 and determined the solubility of the end-modified polymers in the buffer used to dissolve the polymers and form the nanoparticles (25 mM sodium acetate (NaAc) in water). The most hydrophilic polymers (B3-S3-E7 and B3m-S3-E7) were soluble at concentrations exceeding 10 mg/ml, and all polymers tested except for B3m-S4-E7 were completely soluble at 5 mg/ml in 25 mM NaAc (Table S6).

Effect of End-modification—End-modification of each polymer backbone significantly modulated its transfection efficacy and toxicity. Polymers formulated with the E9 end group exhibited significant toxicity, almost regardless of the base polymer they were reacted with. Polymers containing E10 and E12 tended to show low transfection efficiencies with a few notable exceptions (B5-S5 and B6-S5 base polymers end modified with E10 and E12 showed transfection efficacies comparable to commercially available transfection reagents LipofectamineTM 2000 and FuGENE[®] HD).

To look at how changing side chains affected the performances of all polymers containing a particular end group, we collapsed log-scale luminescence data for all polymers containing the same backbone monomer together into one composite value (Figure 7). Among the side chains tested, polymers containing the most hydrophobic amino alcohol (S5) were in general the most effective for transfection of COS-7 cells ($p < 0.01$ vs S4, $p < 0.001$ vs S3, S3o, 1-way ANOVA with Tukey's multiple comparison test) and polymers containing the S4 and S3 side chains were significantly more effective than those containing the S3o side chain ($p < 0.01$ vs S4, $p < 0.05$ vs S3o).

To look at structure-function relationships within the polymer library, using a non-parametric correlation (Spearman), we calculated correlation coefficients and two-tailed p-value comparing each end-modifying amine (Table S7). The most structurally similar end-modifying amines were also the most highly correlated in this analysis. Performance of polymers containing E1 and E3 end groups (both diamines separated by 3 carbons) was highly correlated ($R = 0.951$, $p = 1.4 \times 10^{-10}$); performance of polymers containing E6, E7 and E8 (one primary amine, one or two secondary/tertiary amines) were also highly correlated (E6 to E8: $R = 0.950$, $p = 6.3 \times 10^{-10}$; E6 to E7: $R = 0.921$, $p = 8.4 \times 10^{-9}$; E7 to E8: $R = 0.918$, $p = 1.23 \times 10^{-8}$). Interestingly, the performance of polymers containing E3 was also highly correlated with the performance of polymers containing E6 and E8 (E3 to E6: $R = 0.930$, $p = 2.8 \times 10^{-9}$; E3 to E8: $R = 0.928$, $p = 3.6 \times 10^{-9}$).

With same base polymers such as B6-S3, it was interesting to find that end-group could significantly modify the overall efficacy from nearly no expression when end-modified with E12, to increased expression that was over 3 log orders higher with B6-S5-E5 and E6, even though neither end-modified polymer was very toxic (Figures 3 and 4). This effect is also seen across the whole library. If we average the log-scale luminescence seen with all polymers with the same end-modifying amine (Figure S4), we find that all polymers modified with E9 were worse than all other end modified polymers ($p < 0.001$, 1-way ANOVA with Tukey's multiple comparison test), and that E5-end modified polymers were better than E12 end-modified polymers ($p < 0.05$, 1-way ANOVA with Tukey's multiple comparison test). On average, E5 polymers were over 1-log order of magnitude more effective than E12-modified polymers.

To investigate the effects of end-modification further, we synthesized leading end-modified polymers containing all of the diacrylates, the amino alcohol S5, and the end groups E1, E3, E4, E5, E6, E7, and E8, and evaluated their transfection efficacy by flow cytometry at three separate wt/wt ratios (Figure 8, Table S8). For some polymers such as B3m-S5-E5, B3m-S5-E7, B4-S5-E1, B4-S5-E6, B4-S5-E7, B4-S5-E8, etc., optimal transfection occurred at 30 wt/wt, but in almost all of those cases the transfection efficacy did not fall off that significantly from 30 to 60 wt/wt; for some of these, transfection efficacy was relatively consistent in all three formulation conditions, but for others it decreased with increasing wt/wt, often decreasing most dramatically from 60 to 90 wt/wt (3m57, 457, 557). There were also polymers which showed optimal transfection at 60 wt/wt (better than at 30, 90 wt/wt) such as 355 and 454. From a base polymer perspective, B4-S5 was the most efficient (best at 30 wt/wt) and transfection did not drop off with increasing wt/wt ratio. On the other hand, B3-S5 polymers tended to get better at transfection with increasing wt/wt ratio; B5-S5 was optimal at 30 wt/wt and decreased from there; and B6-S5 didn't transfect well at 30 wt/wt but tended to be optimal at 60 wt/wt (and worse again at 90wt/wt). A comparison of the results obtained via the luciferase assay with the results from flow cytometry indicates the same overall pattern, although there are some differences. Similar findings have been shown in the literature, both with similar polymeric systems²⁴ and liposomal systems.³² Differences between in vitro gene delivery assay systems may be due to the fact that they report different things. Luminescence measures a total protein yield per well, whereas flow

cytometry highlights a binary separation of the individual cells within a cell population. In cases where a small percentage of cells express a high level of the exogenous gene or where a high percentage of cells express a low level of the exogenous gene, the assay results will diverge. Leading polymers were thoroughly evaluated by using both of these complementary assay methods.

Generally, polymers were optimal or not significantly suboptimal at 60 wt/wt ratio. The top six polymers overall were B3-S5-E1, B3-S5-E5, B3m-S5-E7, B4-S5-E3, B4-S5-E4, and B4-S5-E7; they transfected COS-7s at the following levels when formulated at 60 wt/wt (RLU, %GFP+): 351 (1.64×10^6 , 64.5%), 355 (2.02×10^6 , 69.4%), 3m57 (2.24×10^6 , 67.2%), 453 (1.97×10^6 , 67.63%), 454 (1.89×10^6 , 76.6%) and 457 (5.63×10^5 , 72.5%). These polymers yielded superior efficacy ($p < 0.001$ for both luminescence and fluorescence via 1-way ANOVA of all polymers and using Dunnet's post test) when compared to the positive controls, FuGENE® HD (7.21×10^5 , 29.9%) and Lipofectamine™ 2000 (8.07×10^5 , 42.9%).

As compared to other non-viral approaches found in literature, optimized PBAE formulations are superior to most alternatives. PEI complexes were reported to yield 29.14% transfection and optimized solid lipid nanoparticles were reported to result in 14.64% transfection with reduced cytotoxicity as compared to PEI.³³ Electroporation has been reported to yield 71.23% transfection.³⁴

In comparing luminescence to GFP assays as measures of gene delivery efficacy, luminescence based assays offer rapid screening options when flow cytometry is either unavailable or cannot be done in a high-throughput fashion, and can quickly give information regarding the overall transfection level. However, it cannot discriminate between a few very bright cells and many moderately transfected cells.

Conclusions

In the study presented here, three hundred and twenty end-modified poly(beta-amino) esters (PBAE) were synthesized and tested for gene delivery efficacy in COS-7 cells. This library approach enabled us to not only synthesize and test a large variety of structures rapidly, but provided us with a reasonably robust dataset to analyze for the effect of small structural permutations to the polymer chain. Most PBAE formulations were optimal at 60 wt/wt (polymer:DNA ratio). Optimal PBAE formulations were superior ($p < 0.001$) to commercially available non-viral alternatives FuGENE® HD and Lipofectamine™ 2000, as they enabled ~3-fold increased luminescence (2.2×10^6 RLU/well vs 8.1×10^5 RLU/well) and 2-fold increased transfection (76.7% vs 42.9%) as measured by flow cytometry with comparable or reduced toxicity. Increasing hydrophobicity of backbone and side chain tended to increase transfection efficacy, and polymers containing the most hydrophobic side chain (S5) and backbone (B6) tended to perform the best. However, increased hydrophobicity of the backbone reduced the requirement for a hydrophobic side chain, and increased hydrophobicity of the side chain reduced the requirement for a hydrophobic backbone, suggesting that there might be some optimal total hydrophobicity for cationic polymer-based gene delivery. End-modification of these polymers produced dramatic results, as differences of greater than 3-log orders of transfection efficacy by luminescence was seen with the same base polymer but with different end-groups. These results taken together suggest that balancing hydrophobicity plays a crucial role in transfection efficacy of these polymers, and that optimized end-modified PBAEs are potentially useful non-viral gene delivery reagents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Check E. Gene therapy put on hold as third child develops cancer. *Nature*. 2005; 433(7026):561.
2. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet*. 2003; 4(5):346–358. [PubMed: 12728277]
3. Check E. A tragic setback. *Nature*. 2002; 420(6912):116–118. [PubMed: 12432357]
4. Gene Therapy Clinical Trials World Wide. <http://www.wiley.com/legacy/wileychi/genmed/clinical/>
5. Sunshine JC, Bishop CJ, Green JJ. Advances in polymeric and inorganic vectors for nonviral nucleic acid delivery. *Therapeutic Delivery*. 2011; 2(4):493–521. [PubMed: 22826857]
6. Putnam D. Polymers for gene delivery across length scales. *Nat. Mater.* 2006; 5(6):439–451. [PubMed: 16738681]
7. Lynn DM, Langer R. Degradable poly(beta-amino esters): Synthesis, characterization, and self-assembly with plasmid DNA. *J Am Chem Soc*. 2000; 122(44):10761–10768.
8. Akinc A, Anderson DG, Lynn DM, Langer R. Synthesis of poly(beta-amino ester)s optimized for highly effective gene delivery. *Bioconjug. Chem.* 2003; 14(5):979–988. [PubMed: 13129402]
9. Yang F, Green JJ, Dinio T, Keung L, Cho SW, Park H, Langer R, Anderson DG. Gene delivery to human adult and embryonic cell-derived stem cells using biodegradable nanoparticulate polymeric vectors. *Gene Ther.* 2009; 16(4):533–546. [PubMed: 19129861]
10. Green JJ, Shi J, Chiu E, Leshchiner ES, Langer R, Anderson DG. Biodegradable polymeric vectors for gene delivery to human endothelial cells. *Bioconjug. Chem.* 2006; 17(5):1162–1169. [PubMed: 16984124]
11. Yang F, Cho SW, Son SM, Bogatyrev SR, Singh D, Green JJ, Mei Y, Park S, Bhang SH, Kim BS, Langer R, Anderson DG. Genetic engineering of human stem cells for enhanced angiogenesis using biodegradable polymeric nanoparticles. *Proc. Natl Acad. Sci.* 2010; 107(8):3317–3322. [PubMed: 19805054]
12. Green JJ, Zugates GT, Tedford NC, Huang HN, Griffith LG, Lauffenburger DA, Sawicki JA, Langer R, Anderson DG. Combinatorial Modification of Degradable Polymers Enables Transfection of Human Cells Comparable to Adenovirus. *Advanced Materials*. 2007; 19(19): 2836–2842.
13. Huang YH, Zugates GT, Peng W, Holtz D, Dunton C, Green JJ, Hossain N, Chernick MR, Padera RF Jr, Langer R, Anderson DG, Sawicki JA. Nanoparticle-delivered suicide gene therapy effectively reduces ovarian tumor burden in mice. *Cancer Res.* 2009; 69(15):6184–6191. [PubMed: 19643734]
14. Sawicki JA, Anderson DG, Langer R. Nanoparticle delivery of suicide DNA for epithelial ovarian cancer therapy. *Adv Exp Med Biol.* 2008; 622:209–219. [PubMed: 18546630]
15. Tseng SY, Guerrero-Cazares H, Martinez EE, Sunshine JC, Quinones-Hinojosa A, Green JJ. Non-viral gene delivery nanoparticles based on Poly(beta-amino esters) for treatment of glioblastoma. *Biomaterials*. 2011; 32(23):5402–5410. [PubMed: 21536325]
16. Lee JS, Green JJ, Love KT, Sunshine J, Langer R, Anderson DG. Gold, Poly(beta-amino ester) Nanoparticles for Small Interfering RNA Delivery. *Nano Letters*. 2009; 9(6):2402–2406. [PubMed: 19422265]
17. Hawkins AM, Milbrandt TA, Puleo DA, Hilt JZ. Synthesis and analysis of degradation, mechanical and toxicity properties of poly(beta-amino ester) degradable hydrogels. *Acta Biomater.* 2011; 7(5):1956–1964. [PubMed: 21252001]
18. Safranski DL, Weiss D, Clark JB, Caspersen BS, Taylor WR, Gall K. Effect of poly(ethylene glycol) diacrylate concentration on network properties and in vivo response of poly(beta-amino ester) networks. *Journal of Biomedical Materials Research Part A*. 2011; 96A(2):320–329. [PubMed: 21171151]
19. Min KH, Kim JH, Bae SM, Shin H, Kim MS, Park S, Lee H, Park RW, Kim IS, Kim K, Kwon IC, Jeong SY, Lee DS. Tumoral acidic pH-responsive MPEG-poly(beta-amino ester) polymeric

- micelles for cancer targeting therapy. *Journal of Controlled Release*. 2010; 144(2):259–266. [PubMed: 20188131]
20. Zugates GT, Tedford NC, Zumbuehl A, Jhunjhunwala S, Kang CS, Griffith LG, Lauffenburger DA, Langer R, Anderson DG. Gene delivery properties of end-modified poly(beta-amino ester)s. *Bioconjug Chem*. 2007; 18(6):1887–1896. [PubMed: 17929884]
21. Sunshine J, Green JJ, Mahon KP, Yang F, Eltoukhy AA, Nguyen DN, Langer R, Anderson DG. Small-Molecule End-Groups of Linear Polymer Determine Cell-Type Gene-Delivery Efficacy. *Advanced Materials*. 2009; 21(48):4947–4951.
22. Bhise NS, Gray RS, Sunshine JC, Htet S, Ewald AJ, Green JJ. The relationship between terminal functionalization and molecular weight of a gene delivery polymer and transfection efficacy in mammary epithelial 2-D cultures and 3-D organotypic cultures. *Biomaterials*. 2010; 31(31):8088–8096. [PubMed: 20674001]
23. Anderson DG, Akinc A, Hossain N, Langer R. Structure/property studies of polymeric gene delivery using a library of poly(beta-amino esters). *Mol. Ther.* 2005; 11(3):426–434. [PubMed: 15727939]
24. Green JJ, Langer R, Anderson DG. A Combinatorial Polymer Library Approach Yields Insight into Nonviral Gene Delivery. *Acc Chem Res*. 2008
25. Liu ZH, Zhang ZY, Zhou CR, Jiao YP. Hydrophobic modifications of cationic polymers for gene delivery. *Progress in Polymer Science*. 2010; 35(9):1144–1162.
26. Gabrielson NP, Pack DW. Acetylation of polyethylenimine enhances gene delivery via weakened polymer/DNA interactions. *Biomacromolecules*. 2006; 7(8):2427–2435. [PubMed: 16903692]
27. Forrest ML, Meister GE, Koerber JT, Pack DW. Partial acetylation of polyethylenimine enhances in vitro gene delivery. *Pharmaceutical Research*. 2004; 21(2):365–371. [PubMed: 15032320]
28. Eliyahu H, Makovitzki A, Azzam T, Zlotkin A, Joseph A, Gazit D, Barenholz Y, Domb AJ. Novel dextran-spermine conjugates as transfecting agents: comparing water-soluble and micellar polymers. *Gene Therapy*. 2005; 12(6):494–503. [PubMed: 15565162]
29. Fortune JA, Novobrantseva TI, Klibanov AM. Highly effective gene transfection in vivo by alkylated polyethylenimine. *J Drug Deliv*. 2011;204058. [PubMed: 21490747]
30. Santos JL, Oliveira H, Pandita D, Rodrigues J, Pego AP, Granja PL, Tomas H. Functionalization of poly(amidoamine) dendrimers with hydrophobic chains for improved gene delivery in mesenchymal stem cells. *Journal of Controlled Release*. 2010; 144(1):55–64. [PubMed: 20138937]
31. Wang BQ, He CB, Tang C, Yin CH. Effects of hydrophobic and hydrophilic modifications on gene delivery of amphiphilic chitosan based nanocarriers. *Biomaterials*. 2011; 32(20):4630–4638. [PubMed: 21440295]
32. Zou S, Scarfo K, Nantz MH, Hecker JG. Lipid-mediated delivery of RNA is more efficient than delivery of DNA in non-dividing cells. *Int. J. Pharm.* 2010; 389(1–2):232–243. [PubMed: 20080162]
33. Doroud D, Vatanara A, Zahedifard F, Gholami E, Vahabpour R, Najafabadi AR, Rafati S. Cationic Solid Lipid Nanoparticles Loaded by Cysteine Proteinase Genes as a Novel Anti-Leishmaniasis DNA Vaccine Delivery System: Characterization and In Vitro Evaluations. *Journal of Pharmacy and Pharmaceutical Sciences*. 2010; 13(3):320–335. [PubMed: 21092706]
34. Parham JH, Iannone MA, Overton LK, Hutchins JT. Optimization of transient gene expression in mammalian cells and potential for scale-up using flow electroporation. *Cytotechnology*. 1998; 28(1–3):147–155. [PubMed: 19003416]

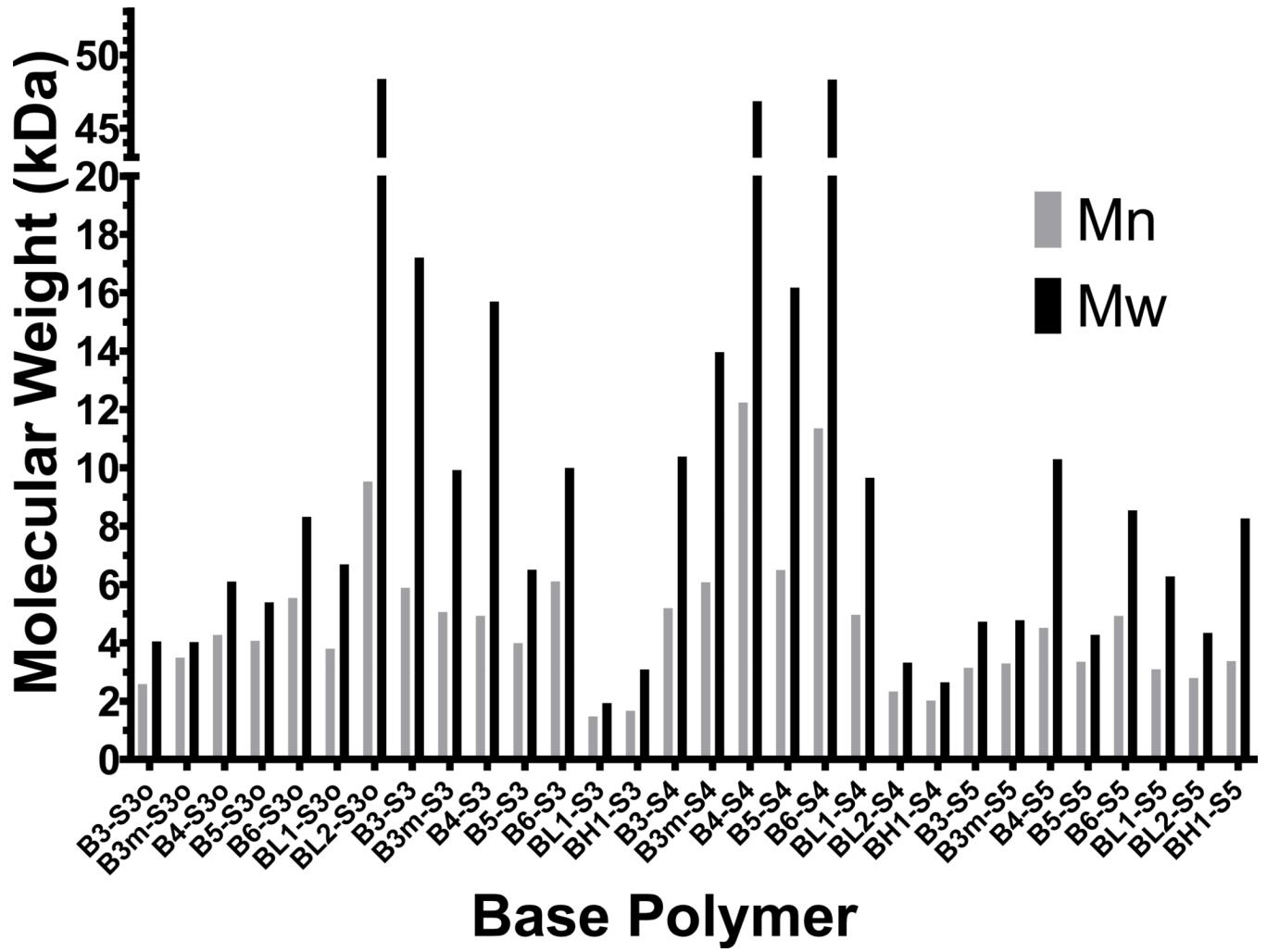


Figure 1.
Base polymer molecular weight by gel permeation chromatography

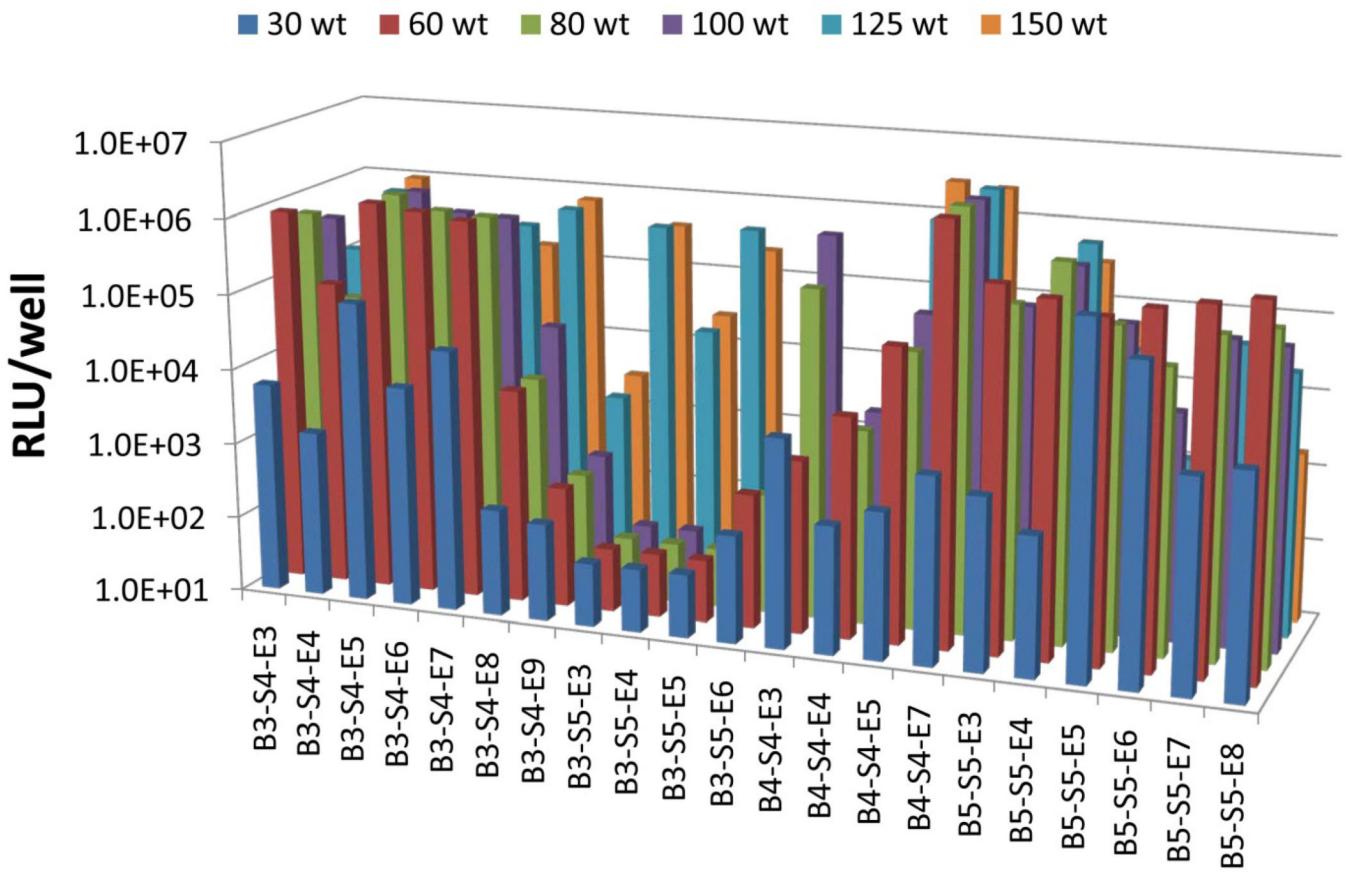


Figure 2.

Transfection efficacy (average RLU/well, n=4) of representative polymers, formulated at a range of polymer:DNA wt/wt ratios. For most polymers, 60 wt/wt was found to be the optimal polymer:DNA ratio.

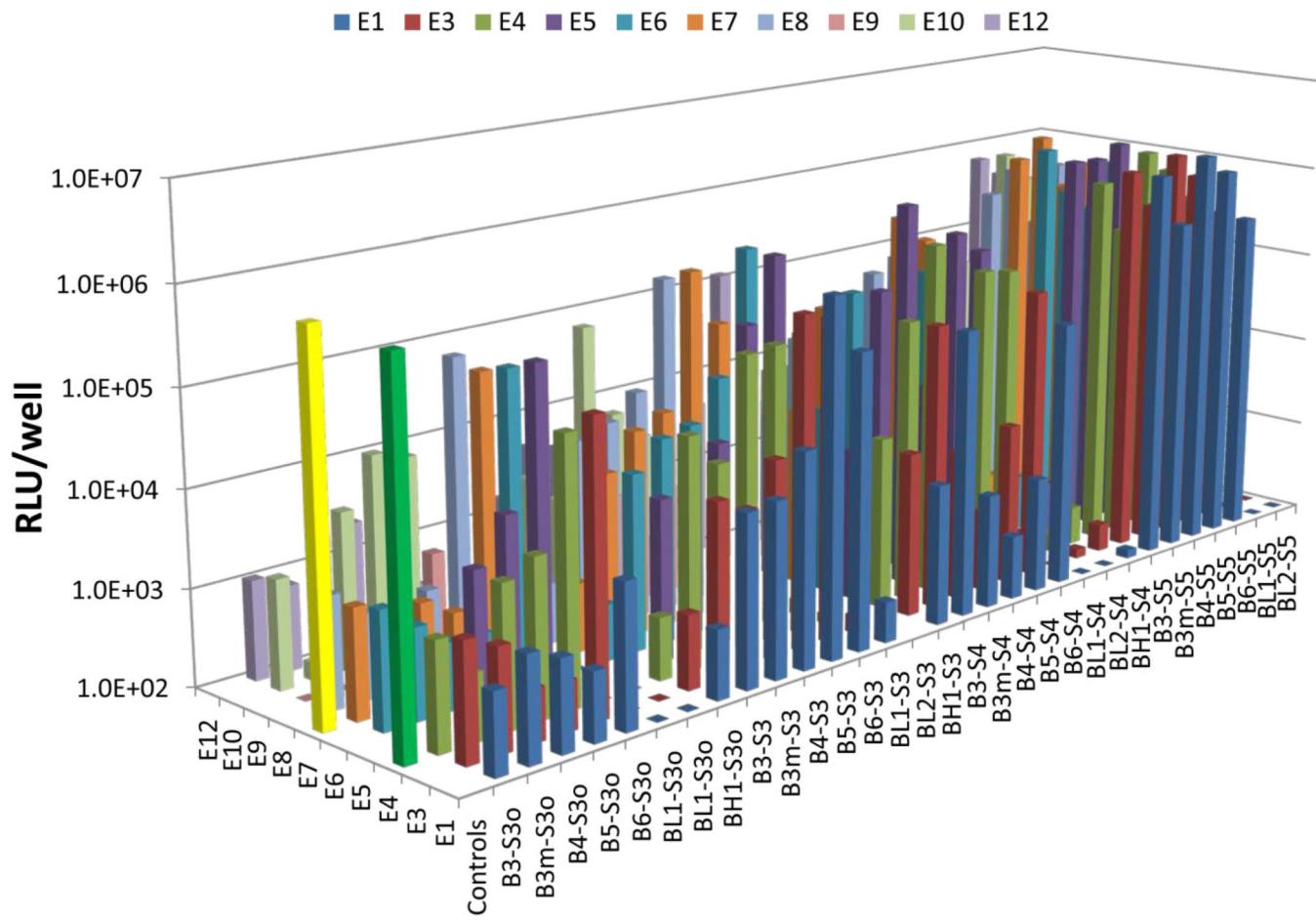


Figure 3.

Average luminescence per well, 48 hours post transfection ($n=4$) with CMV-Luc DNA and polymer library at 60 wt/wt (polymer:DNA). In the control column, the green bar corresponds to FuGENE[®] HD, and the yellow bar corresponds to LipofectamineTM 2000.

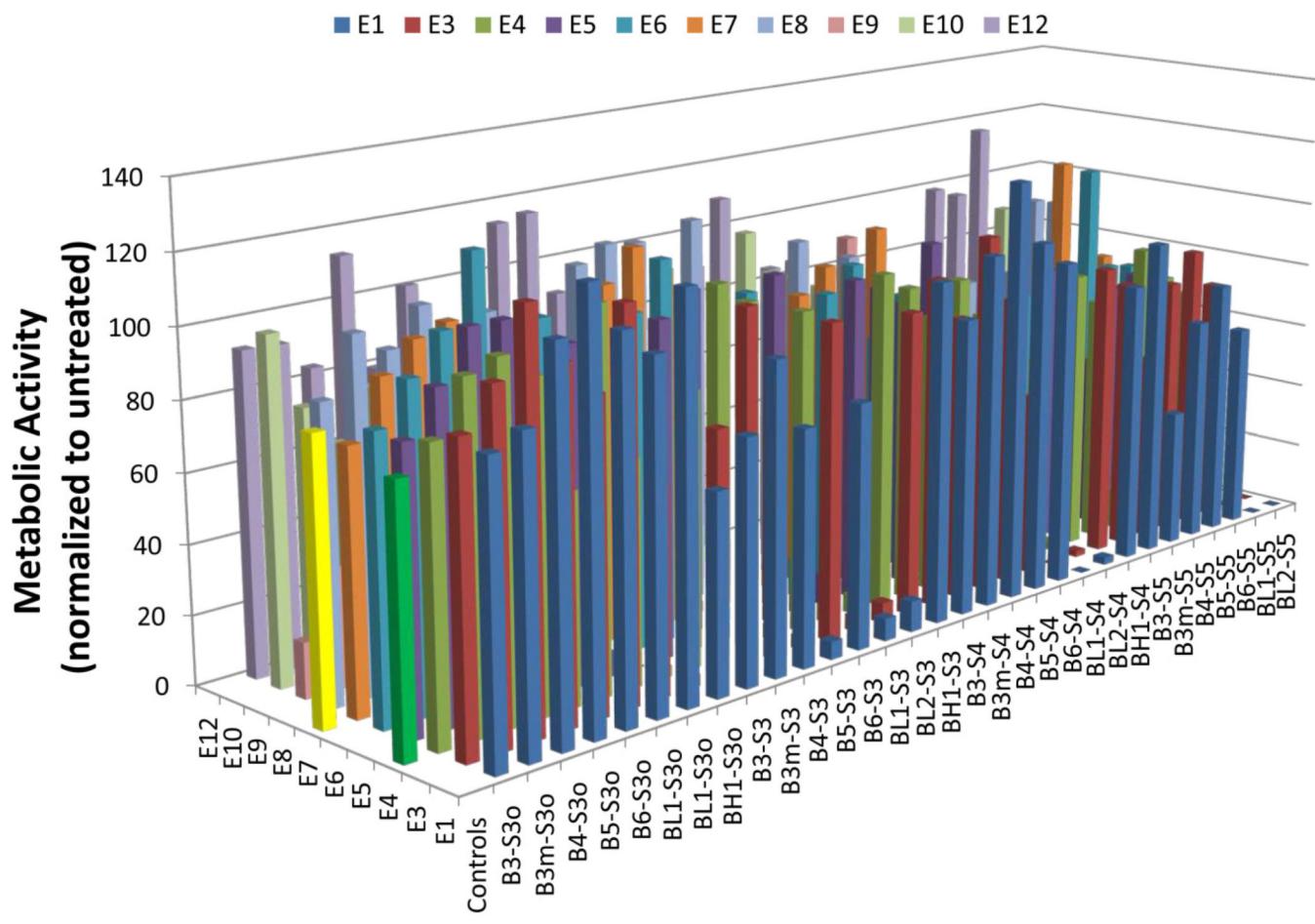
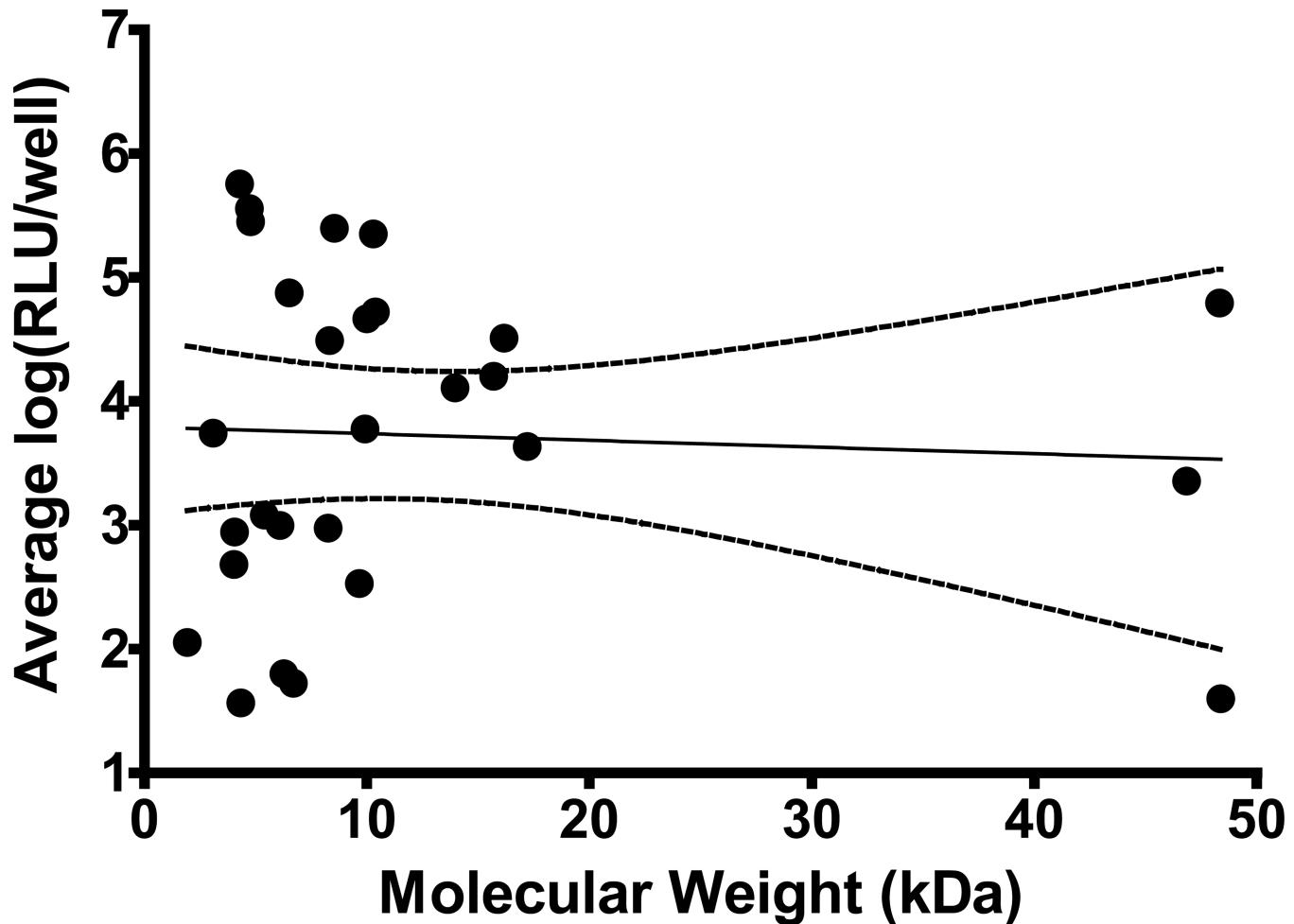
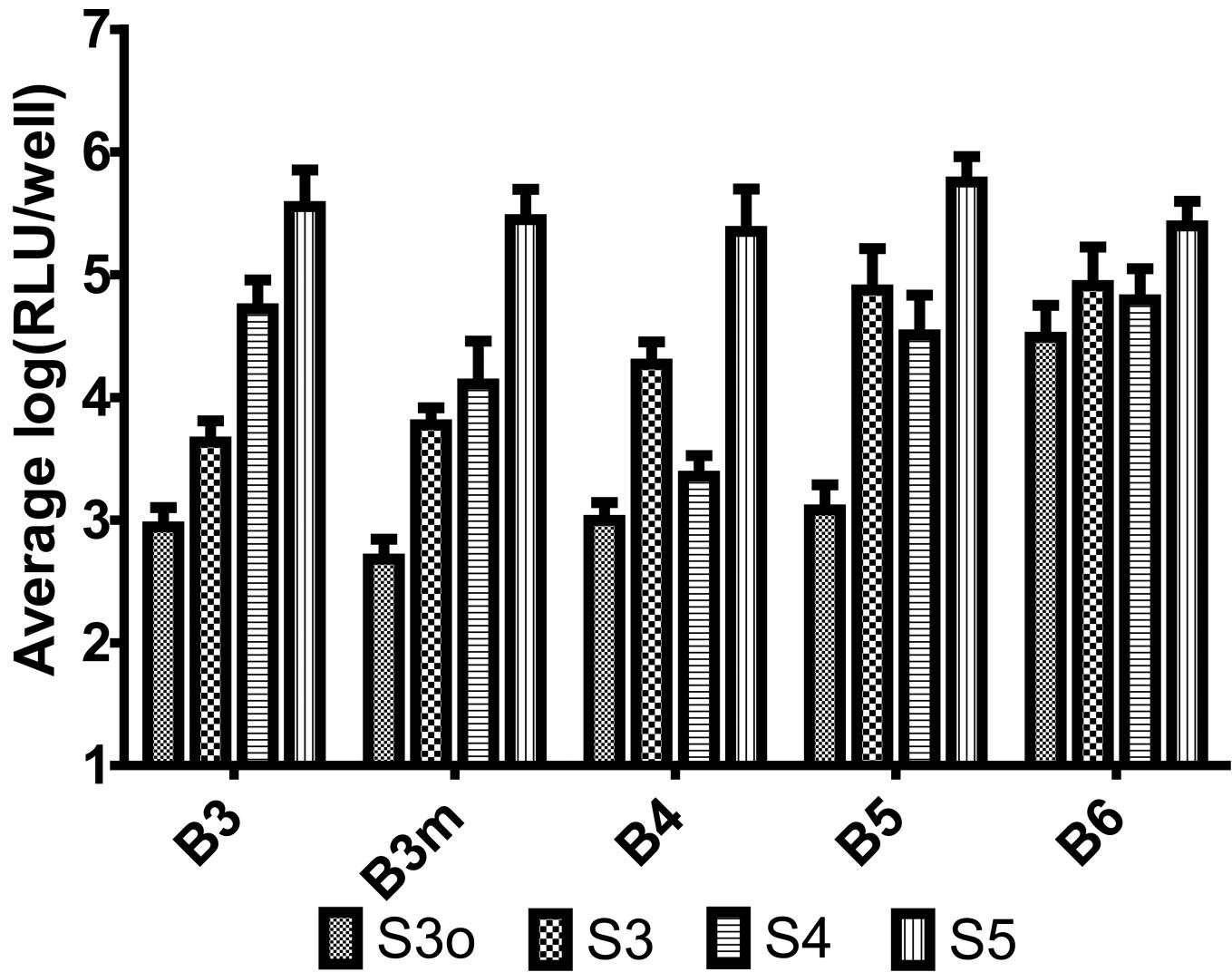


Figure 4.

Metabolic activity of COS-7 cells 24 hours post-transfection (n=4) with CMV-Luc DNA and polymer library at 60 wt/wt (polymer:DNA)assessed by the CellTiter 96® AQ_{ueous} One MTS assay (n=4) and normalized to untreated control wells. In the control column, the green bar corresponds to FuGENE® HD, and the yellow bar corresponds to Lipofectamine™ 2000.

**Figure 5.**

Base polymer molecular weight vs. average log-scale transfection efficacy for all end-modified polymers from the same base polymer. The solid line is the linear regression line ($R^2 = 0.003$, $p = 0.7833$), and the dashed line is the 95% confidence interval of the regression.

**Figure 6.**

Average log-scale luminescence post transfection (mean \pm standard error) of end-modified polymers with the same base polymer, plotted with increasing base diacrylate hydrophobicity. As an example, the far left bar represents the average log-scale luminescence post transfection of all polymers containing the base polymer B3-S3o.

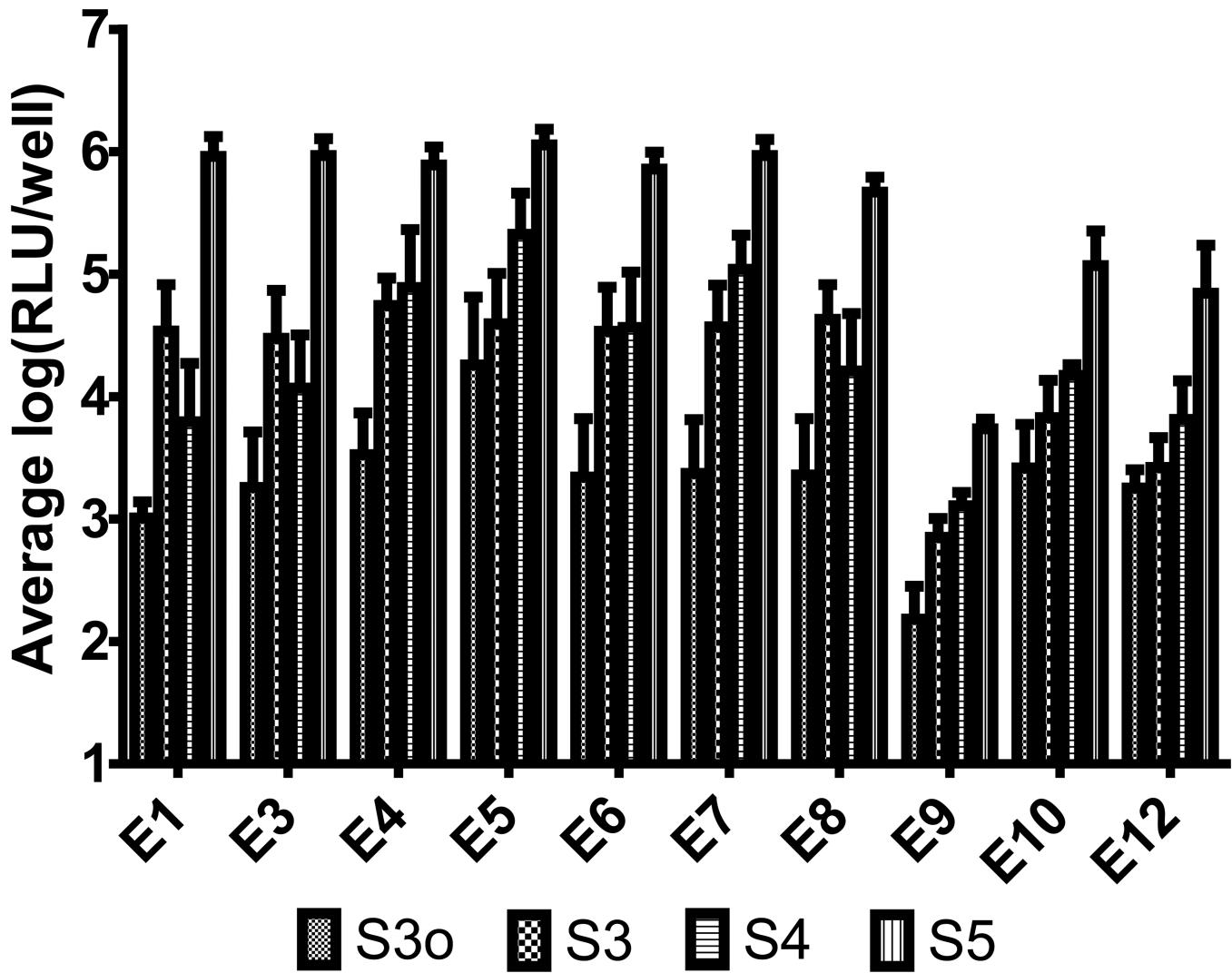
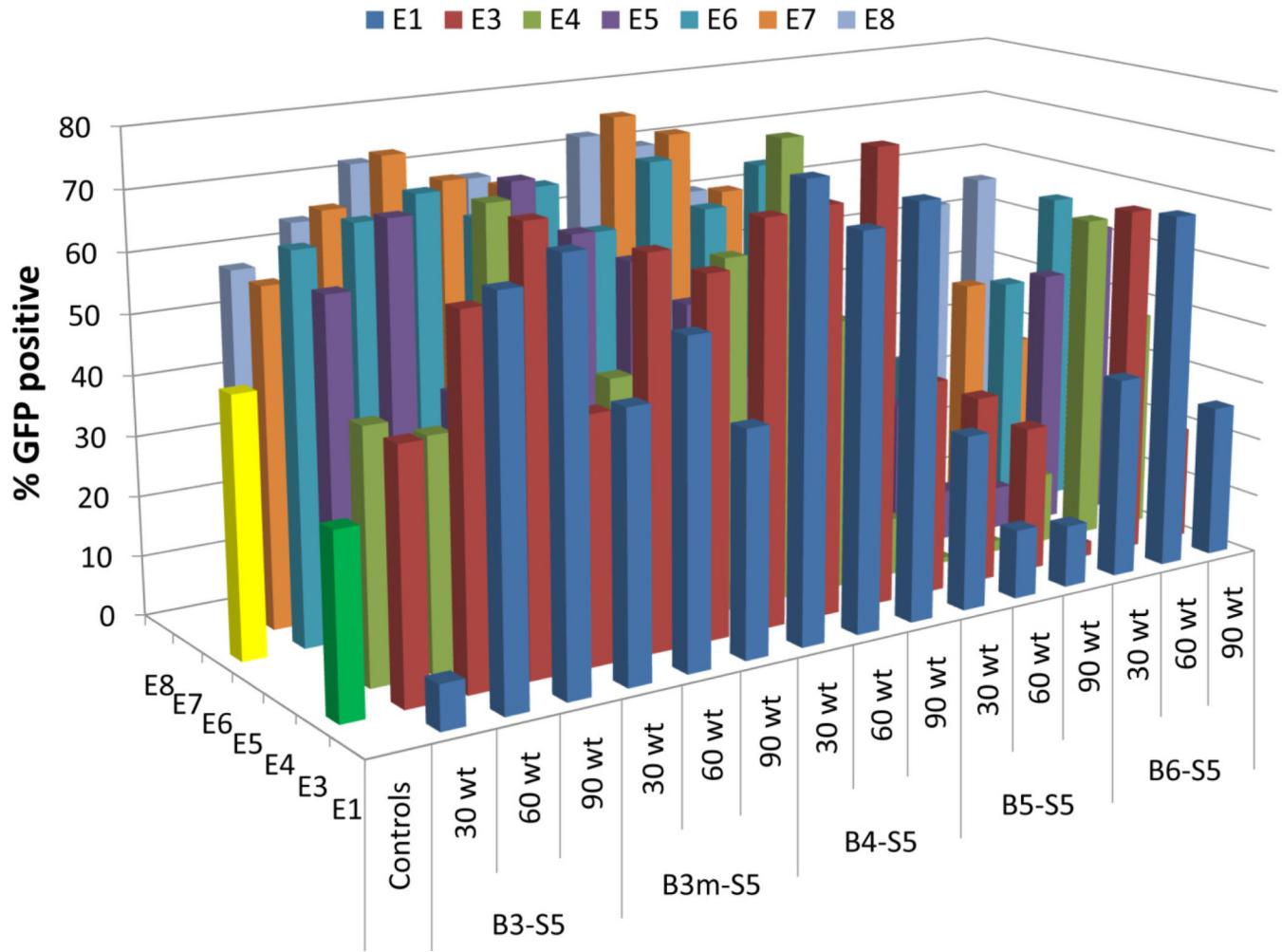
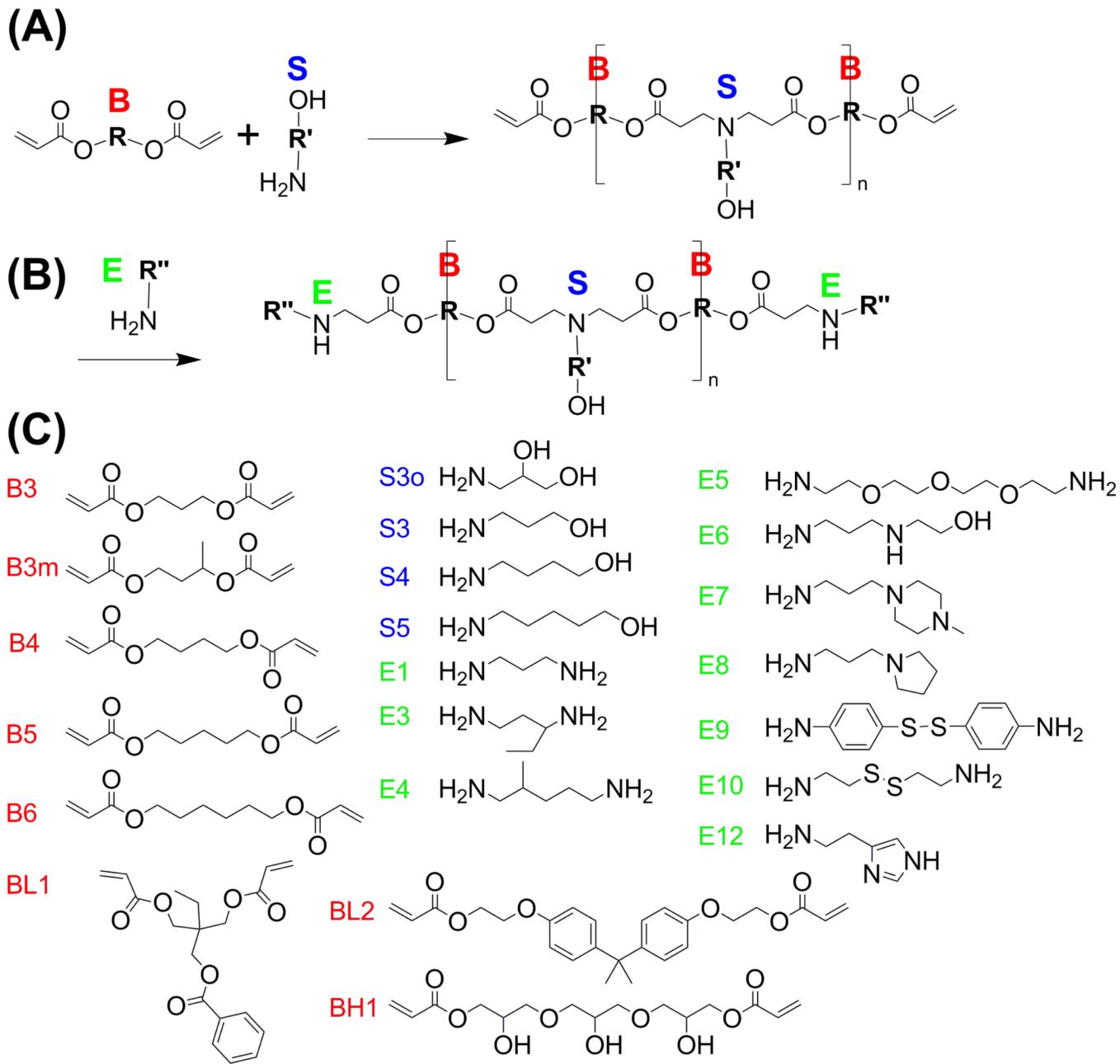


Figure 7.

Average log-scale luminescence post transfection (mean \pm standard error) of polymers containing the side chain and end group listed. For example, the far left bar represents the average log-scale luminescence post transfection of all polymers containing S3o and E1 (B3S3oE1, B3mS3oE1, B4S3oE1, B5S3oE1, and B6S3oE1).

**Figure 8.**

Average transfection efficacy by flow cytometry ($n=4$) of end-modified polymers containing S5. In the control column, the green bar corresponds to FuGENE[®] HD, and the yellow bar corresponds to LipofectamineTM 2000.

**Scheme 1.**

Synthesis of the PBAE library: (A) Diacrylates are reacted with primary amino alcohols by Michael addition with excess diacrylate to form diacrylate-terminated base polymers. (B) Primary amines are added in a second step to form end-modified PBAEs. (C) Library of diacrylates, amino alcohol s, and end-capping amines used in this study are shown.