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Engineering a Polymeric Gene Delivery Vector Based on Poly(ethylenimine) and Hyaluronic Acid

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Abstract

In this work, the effects of primary amines, ligand targeting, and overall charge on the effectiveness of branched poly(ethylenimine)-hyaluronic acid conjugate (bPEI-HA) zwitterionic gene delivery vectors are investigated. To elucidate the relative importance of each of these parameters, the zeta potential, cytotoxicity, and transfection efficiency were explored for a variety of formulations of bPEI-HA. It was found that the length of the hyaluronic acid (HA) oligosaccharide had the most significant effect on cytotoxicity and transfection efficiency with human mesenchymal stem cells. Test groups of bPEI incorporating HA with a length of 10 saccharides had significantly higher transfection efficiency (14.6±2.0%) and lower cytotoxicity than other formulations tested, with the cytotoxicity of the group containing the greatest mass of 10 saccharide showing similar results as the positive controls at the highest polymer concentration (100 μg/ml). Additionally, molar incorporation of HA, as opposed to the saccharide length and HA mass incorporation, had the greatest effect on zeta potential, but a minor effect on both cytotoxicity and transfection efficiency. This work demonstrates the relative importance of each of these tunable design criteria when creating a zwitterionic polymeric gene delivery vector and provides useful specific information regarding the design of bPEI-HA gene delivery vectors.

Keywords

Non-viral gene delivery; ligand targeting; primary amines; hyaluronic acid oligosaccharides; zwitterionic polymer

INTRODUCTION

Therapeutic gene delivery is a developing field that offers an attractive alternative to the direct delivery of therapeutic proteins. Gene delivery opens the possibility of altering cellular protein expression to achieve a therapeutic or otherwise desired response, such as differentiation. The use of non-viral gene delivery vectors mitigates some issues generally associated with viral gene therapy, including limited gene insertion size, immune response, mutagenesis, and large scale production limitations.[1, 2]

Branched poly(ethylenimine) (bPEI) has been shown to be a promising polymeric non-viral gene delivery vector capable of efficient transfection in a number of cell types and situations.[3, 4] A positive aspect of bPEI with relation to the present work is the high density

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of amines and the resulting ease with which chemical alteration can be performed, providing the ability to tailor the polymer for specific applications. For example, in the specific application of tissue engineering, controlled release of gene delivery agents for tissue regeneration can be achieved by loading the gene delivery complexes into implanted carrier scaffolds. To be successful in controlled release, gene delivery systems used in these applications must be able to protect DNA, such that it remains viable through a number of diverse situations that could arise during the construction and loading of the therapeutic carrier scaffolds. Branched PEI systems have shown the capability to withstand multiple preparation methods and retain the viability of DNA for this and other applications.[5, 6]

Although there are several general concerns with the use of bPEI, such as cytotoxicity, previous studies have shown that modification of bPEI can address some of these concerns while improving the transfection efficiency of the vector.[7–19] The incorporation of another molecule, especially a ligand capable of cellular interaction, into the bPEI delivery vector system can be one of the most effective methods of improving the performance of the vector. Several examples of incorporated ligands to allow the targeting of cellular receptors or processes include mannose and other sugars[7–9, 13, 18], folate [17, 19], RGD peptides [14], uronic acids [16], and hyaluronic acid (HA) [10–12, 20, 21]. These previous efforts were designed so that the incorporated ligand would harness the native cellular machinery and increase the cellular uptake of the polymer/DNA complexes, thus increasing transfection efficiency.

Of the many modifications to the PEI system, HA has many promising characteristics that warrant its further use and investigation. HA is a linear non-sulfated glycosaminoglycan with a relatively short *in vivo* half life that is found in essentially every tissue and body fluid in humans.[22] This polysaccharide is a long linear polymer consisting of repeats of the disaccharide β-1,4-glucaronic acid-β-1,3-N-acetyl-D-glucosamine and carries a significant negative charge at physiologic pH because of the carboxyl groups on the polysaccharide chain.[22] Given its ubiquitous distribution, many cell types within the body, including the hMSCs used in this study, have hyaladherins including CD44, CD54, and CD168 designed to bind to HA on the cell surface. [6, 15, 22–29] It is because of these favorable characteristics that the system investigated in this work utilizes the direct conjugation of HA to the bPEI polymer chain for gene delivery.

Previous work involving the modification of PEI with HA has yielded important information regarding the feasibility of creating a zwitterionic polymer capable of transfection.[10, 11, 15] A wide range of MW of HA can be obtained, due to the linear nature of the polymer and the availability of degradation enzymes, and both large and small MW varieties have been investigated for use in a bPEI-HA conjugate system. Generally, the previous approaches can be broken down into two groups. One approach used to create these conjugates focuses on higher MW HA. [20, 21, 27, 28] This approach to conjugation involves activating the carboxyl groups on the HA chain and utilizing these groups to conjugate the amines of bPEI to the large MW HA via an amide bond. In this work involving larger MW HA, the range of bPEI:HA mass ratios explored involved more bPEI than HA within the system. The other main approach to creating bPEI-HA conjugates utilized much smaller HA and a synthesis method based on connecting the primary amines of the bPEI to one of the terminal ends of the linear HA chain via a reductive amination process. [6, 15] This work is the inverse of the large MW HA approach, in that it utilized more HA than bPEI as well as attached the HA to a bPEI backbone rather than bPEI to a HA backbone. The work presented in the present manuscript will continue the development of the latter approach and fully define and test the design parameters that affect the efficacy of the conjugates as transfection agents. By expanding the range of tested HA:bPEI synthesis ratios the work presented in this manuscript will lend greater understanding to both synthesis approaches and will explore the

various design parameters involved in the efficacy of these conjugates with respect to cytotoxicity, transfection efficiency, and zeta potential.

Studies have shown that the negative charges and native nature of HA mitigate the high toxicity commonly associated with the high density of positive charges of bPEI, regardless of HA MW. The present work focuses on the use of relatively low MW HA, which will allow for a broad examination of the polymer conjugate system due to the ability to induce greater variation in amine concentration with less variation in HA weight incorporation. Additionally, HA association with bPEI/DNA complexes can positively alter the packing efficiency and transfection efficiency of the complexes.[10, 11] By combining HA and bPEI, the advantages of each can be used to generate a more efficient vehicle for cellular uptake and gene delivery.[15] However, the relative importance of each of the parameters in this system with regards to cytotoxicity, zeta potential, and transfection efficiency of the complexes are unclear.

The studies presented here focus on the design aspects of engineering a more effective gene delivery vector utilizing bPEI directly conjugated with HA. Specifically, the objective of the present work is to investigate the effects of HA oligosaccharide length and concentration within the bPEI-HA vector on its zeta potential, cytotoxicity, and its ability to transfect human mesenchymal stem cells (hMSCs) *in vitro*. Additionally, by investigating the effects of the experimental parameters explored in this example system, the general mechanisms found in other systems could potentially be elucidated and better understood.

EXPERIMENTAL PROCEDURES

Materials

Chemicals used for the synthesis and purification of HA oligosaccharides, specifically sodium borate, sodium chloride, sodium cyanoborohydrate, sodium acetate, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Hyaluronidase was purchased from Worthington Labs (Lakewood, NJ). HA was purchased from LifeCore Biomedical (Chaska, MN). Branched PEI (MW=25,000) and polyacrylamide bead P2 desalting columns were purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure 2,5 - dihydroxybenzoic acid (DHB) MALDI matrix was purchased from Protea Biosciences (Morgantown, WV). Human MSCs were obtained from the Texas A&M University Cell Distribution Center at the Texas A&M University HSC COM in Temple, Texas. The calcein assay kit was obtained from Molecular Probes (Carlsbad, CA). VivaSpin centrifuge dialysis membranes of 30,000 molecular weight cutoff (MWCO) were obtained from the Sartorius Corporation (Edgewood, NY). Anion exchange columns were obtained from GE Lifesciences (Piscataway, NJ). Cell culture materials included α-MEM, glutamine, trypsin, and phosphate buffered saline (PBS) and were obtained from Gibco (Carlsbad, CA). Plasmid DNA (pDNA) encoding for enhanced green fluorescent protein (eGFP) with the cytomegalovirus (CMV) promoter (pCMV-eGFP, 4.7 kb, cat no. 6085-1) was obtained from Clontech (Palo Alto, CA).

Degradation, Separation, and Characterization of HA Oligosaccharides

HA oligosaccharides were obtained through enzymatic degradation of relatively high molecular weight (MW) HA using hyaluronidase through a previously described process.[30–32] Briefly, a 100 ml solution of 100 mM NaAc and 150 mM NaCl was prepared. 1 g of HA was then added and allowed to dissolve overnight while stirring at 37°C. Once fully dissolved, 100 kilounits (KU) of hyaluronidase were added to the solution and the reaction was allowed to incubate either to completion (overnight) or for 90 min, depending on the size of oligosaccharides desired. At the completion of the degradation time period,

the solution was boiled for 10 min, centrifuged, and the supernatant collected to deactivate and remove the enzyme.

The resulting oligosaccharides in the supernatant were then separated using an anion exchange column (Mono Q 5/50 GL) in a fast protein liquid chromatography (FPLC) instrument. This separation was performed using a simple gradient that ran from 0% to 30% of a 1 M solution of NaCl over 90 min at a flow rate of 1 ml/min with fractions taken every 2 min. After collection, fractions at the same position from multiple runs were concentrated through lyophilization and direct combination of the resulting powder.

Characterization and confirmation of the HA oligosaccharide length was achieved utilizing ^{1}H NMR and MALDI-TOF. ^{1}H NMR spectra were obtained using a 400 MHz spectrophotometer (Bruker Avance 400 – Zurich, Switzerland) with deuterated water as a solvent and internal reference (δ=4.79). All spectra were recorded at room temperature and processed using MestRe-C software (MestReLab Reseach – S.L., Spain). MALDI-TOF experiments were performed using a MS Autoflex MALDI TOF-TOF instrument (Bruker Daltonics – Fremont, CA) with 2,5-dihydroxybenzoic acid (DHB) as a matrix. For mass spectrometry, each fraction was desalted using D-Salt Polyacrylamide 1800 Desalting Columns from Thermo Scientific (Oxford, IL) to reduce adducts in the system.

UV Spectroscopy

UV spectroscopy was used to determine the concentration of the HA fragments after separation. A concentration dependent peak absorbance shift was observed in the 200–250 nm wavelength range for HA. The relation between concentration and peak position was tested using 6.4 kDa HA and confirmed using 0.975 MDa and 1.59 MDa HA over a variety of HA concentrations, salt concentrations, and pH values in a Cary 5000 UV-Vis-NIR spectrophotometer (Agilent Technologies – Santa Clara, CA). Once established, this relation was used to directly determine the HA concentration in the fractions collected from the above mentioned HA purification process. HA oligomers with a length of 2 saccharides were observed to deviate from the established optical relationship and thus were desalted, lyophilized, and directly massed on an ultrasensitive scale (Mettler Toledo AX105, Columbus, OH) to ensure that the correct amounts were used in subsequent steps.

Synthesis of bPEI-HA

Synthesis of bPEI-HA was achieved utilizing a previously described reductive amination reaction.[15] Briefly, HA and bPEI were added in the desired ratios to a 0.1 M sodium borate buffer in the presence of an excess of the reducing agent sodium cyanoborohydrate. This mixture was held at 42°C for 120 hours to allow the reaction to reach the maximum possible attachment of the HA chains to the primary amines of the bPEI. The resulting product was dialyzed and resuspended with deionized water three times according to the manufacturer's protocol in a VivaSpin centrifuge dialysis tube with a 30,000 MWCO to remove salts and all unreacted products. The recovered bPEI-HA was lyophilized, weighed, and used to complete the ensuing studies described below. To verify the presence and ratios of bPEI and HA in the resulting product, ^{1}H NMR was performed at room temperature in the 400 MHz spectrophotometer with deuterated water as a solvent and internal reference (δ=4.79). All spectra were recorded at room temperature and processed using MestRe-C software. By integrating and comparing the peaks associated with either HA or bPEI within the polymers, the ratio of bPEI to HA in each polymer was ascertained.

Assembly of Polymer/DNA Complexes

To form the polymer/DNA complexes, the procedures outlined previously were followed.[15] Briefly, each of the bPEI-HA groups was dissolved in PBS at a concentration of 1 mg/ml

and filtered through a 0.2 μm filter for sterilization. This solution was then brought to a temperature of 37 °C and a pH of 7.4 and allowed to sit overnight. The following day, DNA encoding for eGFP was separated into 50 μl aliquots for each experimental group, with each aliquot containing the same amount of DNA. Each prepared bPEI or bPEI-HA polymer solution was then added dropwise to the prepared DNA solution such that the N:P ratio was maintained at 7.5:1 for each case. Once the addition was complete, the samples were immediately vortexed, centrifuged, and incubated for 2 hours at room temperature to allow for complete complexation.

Zeta Potential and Dynamic Light Scattering

The zeta potential of polymer/pDNA polyplexes was determined for each group at a constant N:P ratio of 7.5:1, which has been determined to be optimal for bPEI-HA in previous work. [15] Size and zeta potential for each group was tested according to previously established procedures.[33] Briefly, a Zen 3600 Zetasizer from Malvern Instruments (Worcestershire, U.K.) was used to measure the dynamic light scattering and electrophoretic mobility of the polyplexes at 25°C. Size was recorded and the zeta potential was calculated using the Smoluchowski equation and measured values.[34] Zeta potential measurements were performed in 10 rounds of 10 samples, while DLS measurements were performed in 10 runs of 10 samples.

Cytotoxicity Studies

Human mesenchymal stem cells (hMSCs) were expanded and cultured according to the procedures established by Prockop et al.[35] All cells were passage 4 or lower when utilized in this work. To determine the cytotoxicity of each distinct group of bPEI-HA relative to each other and to unmodified bPEI, hMSCs were plated onto 96 well plates at a density of 40,000 cells/cm^2 and allowed to attach overnight. The cells were then exposed to bPEI or bPEI-HA in solution at one of three concentration levels of 10, 50, or 100 μg/ml for 24 hours. Polymer solutions were prepared by dissolution in α-MEM at the appropriate concentrations and filtration through a 0.2 μm filter for sterilization. After 24 hours of exposure, the solution was removed and the cells were rinsed twice with PBS and were analyzed for viability using a calcein assay for mammalian cells from Molecular Probes (Carlsbad, CA). Calcein was selected over other testing methods because of its similar reliability coupled with the ability to visualize the cytotoxicity through fluorescence.[36] To complete the cytotoxicity assay, cells were incubated with 2 μM Calcein-AM for 30 min. For these studies, untreated hMSCs served as live controls, while hMSCs exposed to 90% ethanol for 15 min served as negative controls. Fluorescence of the Calcein-AM was measured for each group using a fluorescence microplate reader (FLx800 Bio-TEK Instruments) equipped with a 485/582 filter (excitation/emission) to measure calcein fluorescence. Live cell fraction was determined in the manner described by Temenoff et al.[37] and six samples were tested for each group.

Transfection Efficiency

For transfection efficiency, hMSCs were expanded and plated onto 6 well plates at a density of 5,000 cells/cm^2. These cells were allowed to attach overnight in the presence of complete medium (500ml α-MEM, 100ml Fetal Bovine Serum (FBS), 6ml L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin). After attachment, the cell cycles were synchronized through incubation in α-MEM medium without the presence of serum for 30 hours; the doubling time of hMSCs.[38] Though it was not anticipated that cell synchronization would have an effect on transfection efficiency, cell cycles were synchronized to mitigate potentially confounding factors. Once synchronized, the cell cycles were restarted by incubation in complete medium for 6 hours.

Transfection was then performed on the prepared cells with the above prepared complexes as previously described.[15] Briefly, complexes were added dropwise to each test well to achieve a final DNA concentration of 50 μg/ml. The controls consisted of untreated cells and cells treated with DNA only. Each group was cultured for 72 hours after exposure to the complexes and then analyzed for transfection efficiency through flow cytometry. Six samples were tested for each group.

To prepare the samples for flow cytometry, cells were washed three times with PBS to remove any dead cells or debris. The remaining live cells were then trypsinized and collected. After collection, the cells were fixed through exposure to a chilled 4% formaldehyde solution for 1 hour. After fixation, the cells were again washed with PBS before being analyzed with a flow cytometer (Becton Dickenson FACS Scan) under high flow rate using the CellQuest Pro software from BD Biosciences (San Jose, CA). PBS containing untreated cells was run through the cytometer to set the gates used to identify the cells on the output graph. The cytometer was set to identify green fluorescence and the total number of cells flowing through it. A limit of 5,000 cells was set for each group, and, in the cases of two of the bPEI only samples, where toxicity was too high, readings were terminated after three min with no new data points. Finally, to account for the population of native cells with rightward shift, markers were placed at 1% of the control samples.

Statistics

Statistical analysis was performed on the data collected for zeta potential, DLS, transfection efficiency, and cytotoxicity using ANOVA with a p value < 0.05. Post hoc analysis was performed via Tukey-Kramer HSD to identify statistical significance between each of the groups.

RESULTS AND DISCUSSION

HA Degradation and Characterization

HA degradation, characterization, and quantification were successfully performed according to the procedures outlined above. Degradation of larger HA chains by hyaluronidase occurs through hydrolysis of the endo-N-acetylhexosaminic bonds between the HA base saccharides and results in HA oligomers. Complete degradation (overnight) of HA results in 2 saccharide HA, while incomplete degradation results in a mixture of oligosaccharides of varying lengths. With incomplete degradation, a longer degradation time results in a broad distribution of HA oligomers, which has a relatively lower average mass. Previous work has shown that a 90 min degradation time resulted in the best distribution of HA oligosaccharides of differing lengths for this work.[30] The oligosaccharide mixture generated by degradation was separated utilizing anion exchange chromatography, resulting in the purification of samples with average lengths of 10 and 16 saccharides, as determined by mass spectrometry. Each of these fractions was then successfully quantified for use in further studies by utilizing the relationship between peak position and HA mass shown in Figure 1. The identification of this relationship between peak absorbance wavelength and concentration allowed for the rapid identification of concentration without further processing the HA oligosaccharides, and associated sample loss. HA is extremely hydrophilic and the identification of this relationship could potentially provide another method to quantify HA that has not been completely dried.

bPEI-HA Synthesis

In this work, 12 distinct formulations of bPEI-HA conjugates, shown in Table 1, were synthesized and investigated. Through proper experimental design, these 12 groups allow for the relative importance of primary amines, overall net charge within the polymer, and

ligand targeting through HA oligosaccharide length to be identified with respect to designing a cationic gene delivery vector. It is important to note that the secondary and tertiary amines of the bPEI are unchanged by the reaction mechanism employed and thus were not considered as a design characteristic in the present study. Four populations of HA oligosaccharides were tested; 2, 10, and 16 saccharides, in addition to a 1:1:1 molar mixture of the three sizes. The 10 saccharide population was chosen for its ability to interact with the ligands on the cell surface. The 2 saccharide population was selected because it represents complete degradation of the HA with hyaluronidase and is incapable of hyaladherin interaction. 16 saccharide HA was chosen as the largest population because of its relative ease of procurement compared to other larger oligosaccharides coupled with the fact that in the groups containing a mixture of HA, the average molecular weight of the mixture was very similar to the average molecular weight of the 10 saccharide groups. This similarity allows for these groups to be compared and conclusions to be drawn about the relative importance of the oligosaccharide length. For each of these oligosaccharide lengths, three molar levels of incorporation were examined: a low (4.4×10^{-4}), medium (8.8×10^{-4}), and high (13.2×10^{-4}) number of moles of HA per mole of bPEI. These incorporation values were chosen based on multiples of the amount used in previous work that established this vector.[15] By utilizing these HA oligosaccharide populations and amounts, the relative importance of each design characteristic with respect to zeta potential, cytotoxicity, and transfection efficiency was effectively explored for these experimental groups.

After synthesis and purification, each of the designed polymers was chemically verified using ^{1}H NMR. Several representative spectra are shown in Figure 2, and as expected, when the overall amount of HA incorporated into the polymer increases, the peaks representing HA increase in intensity. For each NMR result, regions of the peaks representing the HA (δ=4.0–3.1) in the polymer were integrated and compared to the integrated peaks representing the bPEI (δ=3.1–2.5). This ratio was then plotted against the theoretical mass ratio for each polymer in order to verify each individual synthesis by comparing between synthesis groups. The resulting relationship, shown in Figure 3, correlated well between the groups with an R^2 value of 0.9928, though it is important to note that the relationship between the theoretical mass ratio (HA/bPEI) used for synthesis and the measured NMR integration ratio (HA/bPEI) would be expected to differ slightly for a given group, considering small variations in the efficiency of conjugation. However, the relationship between the two ratios verified that each of the polymers used for further testing was synthesized and provided an easy check for the composition of the polymers.

Cytotoxicity

One of the greatest hurdles generally encountered in the application of bPEI and other highly cationic polymers as gene delivery vectors is their high toxicity.[39, 40] It has been shown that by incorporating negatively charged molecules in a gene delivery system, the overall cytotoxicity can be decreased.[21, 41] In the case of bPEI, a relatively high concentration of positive charges associated with the nitrogen along the polymer backbone is thought to be responsible for the cytotoxicity of the vector. By conjugating a negatively charged polymer to this backbone, some of the positive charge associated with these nitrogen groups can be effectively neutralized.

Cytotoxicity studies were performed on each of the polymer groups to identify the roles of the design parameters on the overall toxicity to hMSCs. Results from each group are presented as a ratio relative to the live controls run with each concentration, such that a value of 1 represents the live control. Although it is anticipated that the cytotoxicity of the complexes will differ from the cytotoxicity associated with the polymers alone, the polymers alone were explored in this case to provide a conservative measure of the cytotoxicity of the system that would be applicable regardless of the nucleotide type or size included in the

system. By identifying the most important parameters with relation to cell survival, a gene delivery vector that maximizes transfection efficiency while minimizing cytotoxicity could be developed. The results of this cytotoxicity study are presented in Figure 4. As expected, pure bPEI was especially toxic to cells at all concentrations with a live fraction of only 0.058 ± 0.010 at the lowest tested concentration. By contrast, at the highest concentration, the group containing high amounts of 10 saccharide HA was not different from the live controls. Interestingly, the High10 group presented a live fraction value of greater than 1, which was unexpected but may reflect the propagation of error inherent in the methodology used to calculate the ratios presented. Collectively, these results clearly indicate that incorporation of HA into bPEI has the potential to significantly decrease cytotoxicity.

By examining how each of the groups performed at the lowest concentration (10 μg/ml), interesting conclusions can be drawn regarding the role of primary amines, HA oligosaccharide length, and overall charge in the cytotoxicity of the conjugates towards hMSCs. At 10 μg/ml, the Low2 and High2 groups do not possess significantly different cytotoxicity from pure bPEI. This result is expected, as only a small number of moles of HA are incorporated into the polymer backbone. Also, at 10 μg/ml, group Med2 is significantly different from raw bPEI and has similar cytotoxicity to the Low2, High2, and Low16 groups. Though important, the differences between bPEI and the Low2, Med2, and High2 groups are minor and reflect the fact that the Low2, Med2, and High2 groups only have small HA mass incorporation. When considered globally within this study, these results suggest that HA mass and negative charge incorporation have a greater impact than primary amine concentration in relation to cytotoxicity. As further evidence for this assertion, the cytotoxicities of the Med16 and HighMix groups are not statistically different. Though these groups have a relatively similar HA mass incorporation, they have very different concentrations of primary amines. These observations establish that the overall negative charge incorporation is more important than primary amine concentration within the polymers. This is expected as the cytotoxicity of bPEI is due to the positive charges interacting with and disrupting the cell membrane.[2, 39]

To elucidate the relative importance of the length of HA oligosaccharide with respect to primary amine incorporation and overall charge, numerous groups can be examined. Groups Med10 versus MedMix and High10 versus HighMix have essentially the same mass incorporation of HA into the polymer conjugates, but groups containing 10 saccharide HA have significantly lower cytotoxicity when compared to their counterparts containing a distribution of HA oligosaccharides. The highest overall viability was achieved with the High10 group, a high level of 10 saccharide HA incorporation, but not the highest overall HA mass incorporation. This result displays the fact that the incorporation of HA in a 10 oligomer size range significantly decreases cytotoxicity and is more important with relation to cytotoxicity than HA mass incorporation, [42, 43] which may indicate that the polymers containing 10 saccharide HA are interacting directly with the hyaladherins on the cell surface instead of the cell membrane. Interaction with native cellular machinery could result in less cytotoxicity than disruption of the cell surface with charged molecules. Considering that the 16 saccharide HA can also interact with the hyaladherins, intramolecular interactions within the polymers due to the various lengths of the linear HA chain could be influencing the cytotoxicity of the polymers. These results demonstrate that the length of HA, or potentially any negatively charged polymer or ligand, is essential to decreasing cytotoxicity and should be taken into consideration when designing and synthesizing zwitterionic polymer conjugates as gene delivery vectors.

Zeta Potential and Dynamic Light Scattering (DLS)

In this work, an N:P ratio of 7.5:1 was selected and has been shown to be effective at achieving transfection.[15] By maintaining a constant N:P ratio, a potentially confounding

factor was removed in the present study, although future work could investigate the effect of this specific parameter on transfection efficiency and zeta potential. Complexes were assembled in 150 mM NaCl. While a higher salt concentration generally yields higher transfection percentages[15], an isotonic 150 mM NaCl solution was used in this work to mimic the conditions potentially found within the body. Each of the synthesized polymers was tested for zeta potential and DLS to determine their charge and size in solution. The results of this can be seen in Figure 5 and Table 2. While some of the results contained high standard deviations, this could be the result of uncomplexed DNA and polymer within the solutions, as well as complex aggregation affecting the distribution of the data. The DLS data generally mirrored the trends found in the transfection efficiency and zeta potential, but did not present statistical significance. Specifically, the Low2, Med2, and High2 groups had the largest size and zeta potentials closest to 0 within the test groups, potentially suggesting ineffective complexation. The sizes of the remaining groups generally followed the trend within each HA type; specifically, that a lower zeta potential resulted in a larger complex size. One unexpected result within the DLS data was the large size observed for the bPEI only complexes, as it was expected that the complex size should be smaller than the bPEI-HA polymer complexes. The larger size observed with DLS for the bPEI complexes could reflect potential aggregation within the solution prior to testing, although aggregation was not directly assessed. By contrast, the zeta potential results were helpful in gaining a greater understanding of the system.

As expected, the overall charge of bPEI/DNA complexes was positive due to the excessive positive charge relative to the negative charges along the DNA backbone. The data demonstrate that there is a point of HA mass incorporation for these bPEI-HA conjugates at which the net overall charge of the polymer/DNA complexes in solution becomes negative. The negative zeta potential in solution suggests that, when complexed, HA is present at the surface of the complexes. This is most likely due to the high hydrophilicity of the HA at the temperatures tested relative to bPEI and the fact that the open primary amines will associate with the negative charges present in the complexes and the HA. Also, interestingly, the differences in zeta potential between the Low10, Med10, and High10 groups and the LowMix, MedMix, and HighMix groups are not significant among mass matched sets. This suggests that the complexes have a similar net charge in solution regardless of the HA oligosaccharide length that was conjugated to the bPEI backbone. This lends credence to the theory that ligands on the cell surface are being targeted and are responsible for the increased transfection efficiency and decreased cytotoxicity. In addition to this effect, an HA incorporation dependent charge difference can be seen among groups incorporating the same length of HA oligosaccharide. The data suggest that the medium level of HA incorporation results in the greatest net negative charge of polymer/DNA complexes. This is further confirmed by examining the High10, Med16, and HighMix groups. All three of these groups have a similar mass incorporation, but the Med16 group, which has fewer overall moles of HA present has a significantly more negative zeta potential. It is hypothesized that this effect is due to two competing forces within the polymers, namely primary amine concentration and overall charge. In the case of the lowest molar ratio of incorporation, the primary amine concentration is relatively high while the overall incorporation of negative charges (HA) is relatively low. This leads to a relatively more neutral zeta potential because the DNA is efficiently packed and the low negative charges are present on the surface. In the case of the medium level of HA, the packing efficiency is lower, but the interactions are still strong enough to be effective due to a significant amount of primary amines that remain available for interaction with the DNA backbone. The HA in these polymers is forced to the surface due to this packing combined with the native hydrophilicity of HA. In the case of high incorporation, interactions between the DNA and polymer are weaker due to decreased amine availability, resulting in interactions within the zwitterionic polymers that decrease the negative charges present at the interface of the molecule and water.

Transfection Efficiency

Testing the overall transfection efficiency of each of these polymer groups allows the effects of each design parameter to be elucidated and a cohesive picture to form with respect to the potential effectiveness of these polymers. Each of the polymers was tested and the results are presented in Figure 6. The most effective transfection was achieved with the Low10 (12.15 ±1.46%), Med10 (13.38 ± 5.69%), High10 (14.58 ± 2.01%), Med16 (11.86 ± 0.77%), and MedMix groups (14.43 ± 0.85%).

HA oligosaccharide length is the major design factor that influences transfection efficiency. The results for the Low10 and High10 groups, when compared to the LowMix and HighMix groups, clearly indicate that the groups incorporating 10 saccharide HA yield significantly higher transfection than groups incorporating other lengths of HA. Though this could be an effect of selecting an HA polymer of a length that allows for the optimal interaction with the DNA or bPEI, this could be also be due to the potential for the 10 saccharide HA to interact with hyaladherin ligands on the cell surface. By harnessing the cell machinery already designed for the uptake of HA from the extracellular environment, cellular uptake could be improved with these polymers and transfection increased. It is important to note that the 16 saccharide HA should also be available to interact with the HA cell surface receptors, and this can be seen in the results of the Med16 group. The relatively low transfection efficiency observed for the High16 group could be due to the high amount of HA incorporation for this group, which could cause instability within the polymer/DNA complexes. The previous results from cytotoxicity and zeta potential could suggest that there is a ligand-mediated effect, but it is probable that the improved transfection efficiency is due to a combination of length and hyaladherin targeting. However, elucidation of the mechanism of interactions associated with the transfection observed with each group was beyond the focused aims on the present study.

In addition to these potential ligand interactions, there is a range of molar incorporation of HA conjugate that should be included into the system to generate more effective transfection. The groups incorporating a medium number of moles (8.8×10^{-4} moles of HA per mole of bPEI) of HA into the bPEI backbone resulted in significantly higher transfection in each group of HA investigated, with the exception of 10 saccharide HA. This shows that an appropriate balance must be realized within the molecules to allow for efficient cell association, complex uptake, and endosomal release. The zeta potential roughly mirrors the transfection efficiency and the rationale behind the results is similar. These results suggest that some of the HA is present on the surface of the complexes and is contributing to both the increased uptake and negative zeta potential. The medium HA incorporation ratio balances the primary amines in the system such that packing occurs but is not too tight to limit efficient unpacking and dissociation, and thus transfection.

Least important to transfection is the overall HA charge incorporation. The data from the Low2, Med2, and High2 group show that a low incorporation of HA mass results in significantly decreased transfection efficiency. On the other hand, once a threshold incorporation of HA mass is reached, further incorporation does not have a major effect on transfection. This effect is reflected in data from the High10, Med16, and HighMix groups. Specifically, while each of these groups has a similar mass incorporation, the transfection efficiency is far from uniform, with the HighMix group being significantly lower. It is hypothesized that this is because the conjugate selection is most important for association of the complexes with the cell surface and amine concentration is most important in endosomal buffering and escape, as has been indicated in the literature[44]. These two major factors mask any effects that charge would have on the transfection efficiency.

It is important to note that the above conclusions and considerations were formed using plasmid DNA encoding for eGFP. It is hypothesized that in cases utilizing other types or sizes of nucleic acids, the results would be very similar. Because the vector was tested for cytotoxicity in the absence of nucleic acid, the trends and conclusions here should be conserved with the application of this vector towards other lengths and types of nucleic acids. The identification of ligand effects in these results suggests that the polymers themselves, rather than the polymer/DNA complexes, are significantly different from each other and native bPEI with respect to their effects on cells. With this in mind, one could assume that the differences observed with these polymers would extend to other types and lengths of nucleotides. If cells lacking hyaladherins on their surface were to be targeted with these polymers, the results might not be as significant in the group that contained 10 saccharide HA. In these cases, the other conclusions that were drawn regarding primary amine concentration and overall charge incorporation should remain valid.

CONCLUSION

The results presented here demonstrate that the selection of HA oligosaccharide for incorporation into bPEI-HA polymers has the greatest effect on the transfection efficiency and cytotoxicity of bPEI-HA polymers. Specifically, use of 10 saccharide HA with these polymers significantly increases the transfection efficiency and decreases cytotoxicity when used with hMSCs. Also important in the system is the primary amine concentration of the polymer. A medium level of primary amines incorporated into the polymers yields higher transfection efficiency, while greater HA incorporation, and thus overall negative charge, lowers cytotoxicity. These results confirm that use of bPEI-HA yields improved gene delivery vector characteristics over bPEI alone and further illuminates the relative importance of primary amine concentration, overall charge incorporation, and HA oligosaccharide selection when designing these polymers.

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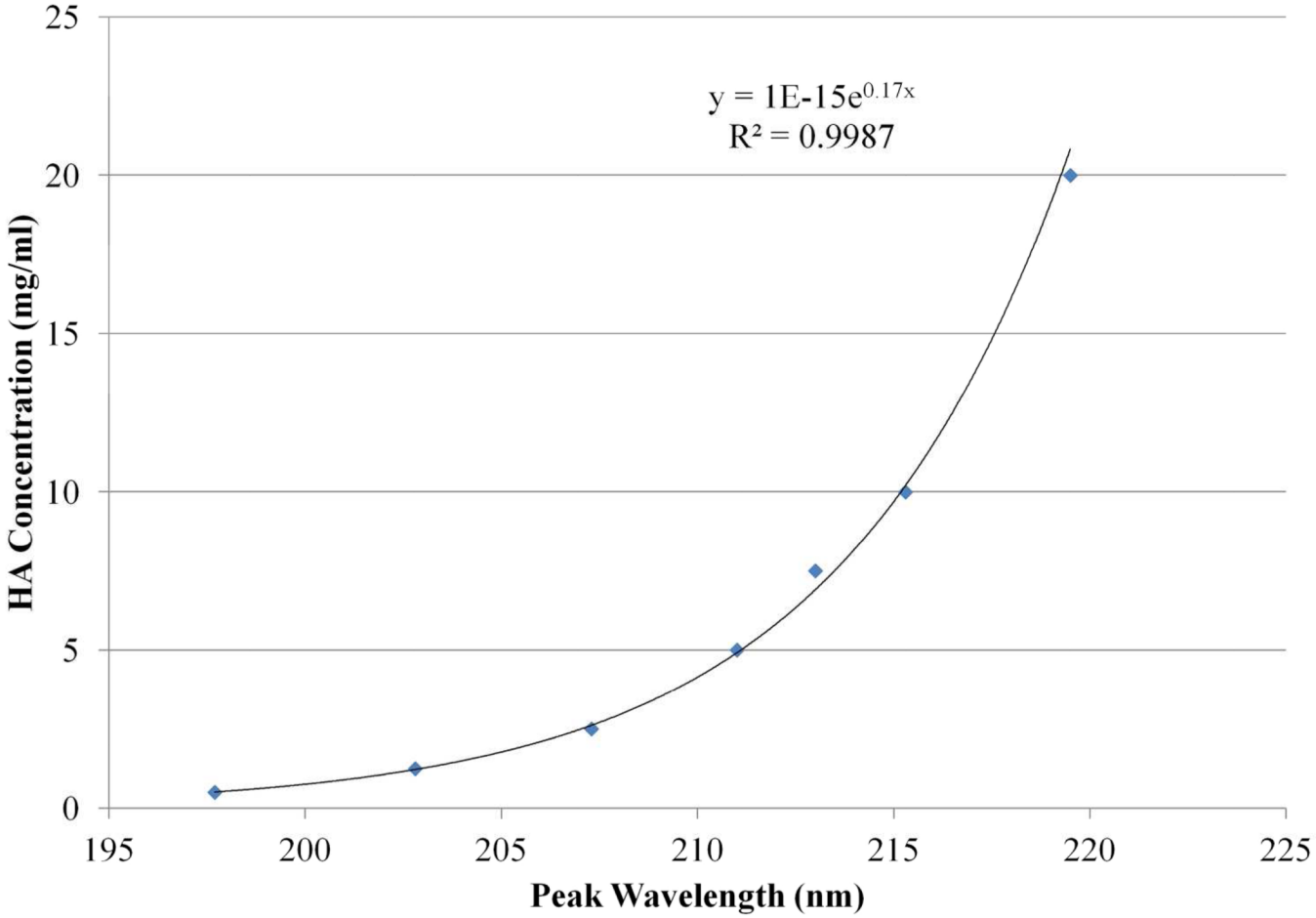

Figure 1.
Graph demonstrating the concentration dependent HA peak shift. This curve shows an exponential relation between the concentration and peak absorption wavelength.

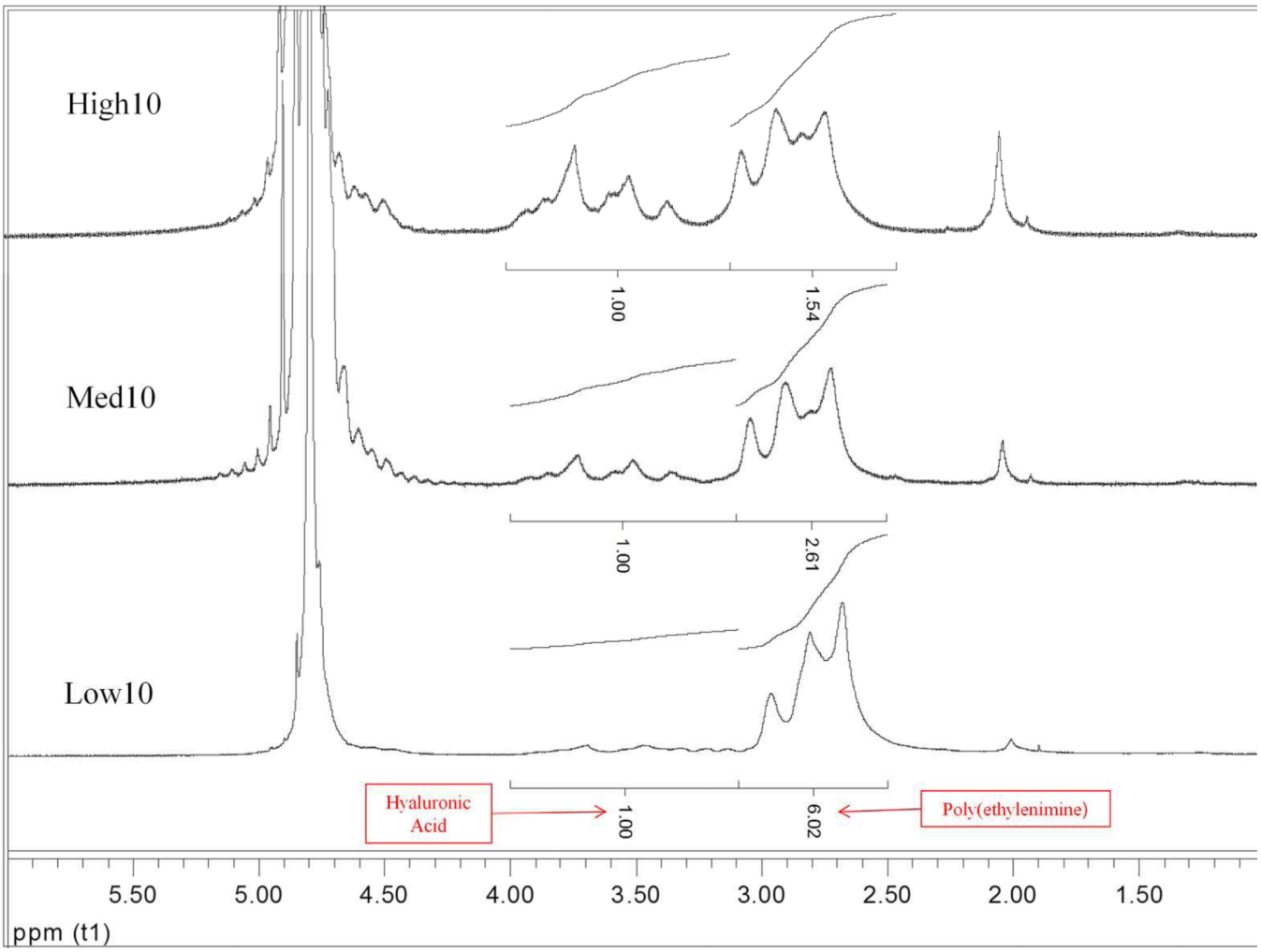

Figure 2.
Representative ^{1}H NMR spectra that were used to verify the relative ratios of bPEI and HA in each synthesized polymer.

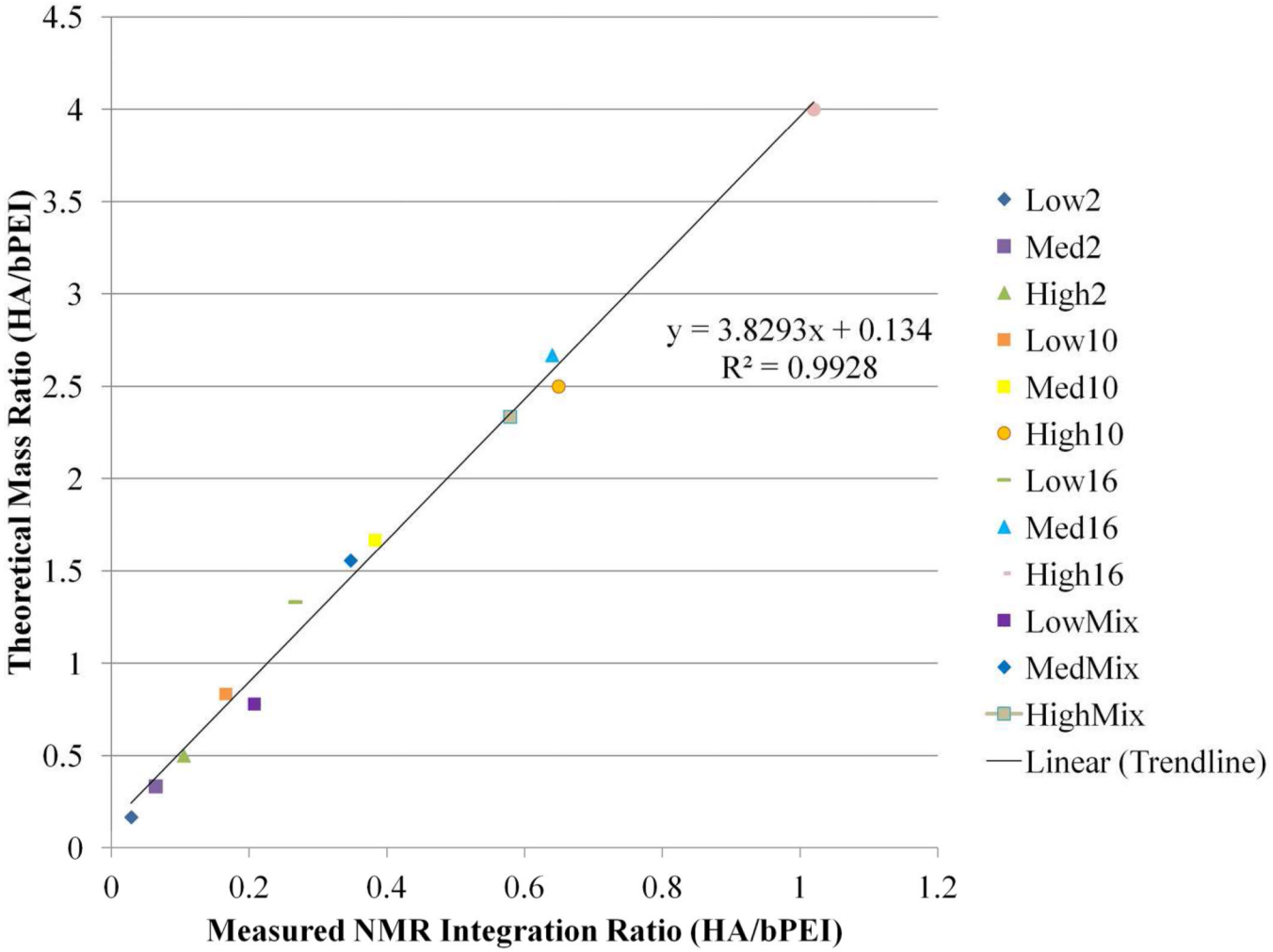

Figure 3.
Confirmation of actual synthesized bPEI-HA ratios through comparing theoretical synthesis ratios with the measured ^{1}H NMR ratios found from integration of the HA and bPEI peaks after synthesis.

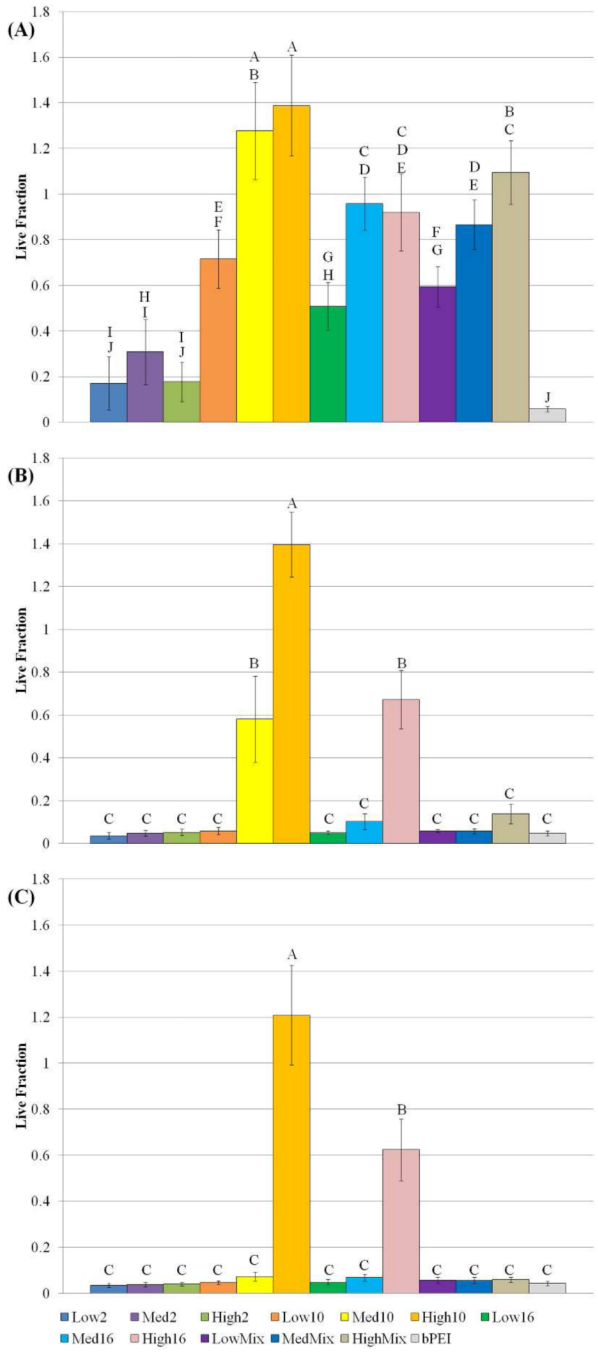

Figure 4.

Cytotoxicity values for each bPEI-HA conjugate. Cells were exposed to each group of bPEI-HA with concentrations of (A) 10 μg/ml, (B) 50 μg/ml, and (C) 100 μg/ml for 24 hours and tested using a Calcein assay. Error bars represent 1 standard deviation, and groups that share letters are not significantly different ($p<0.05$).

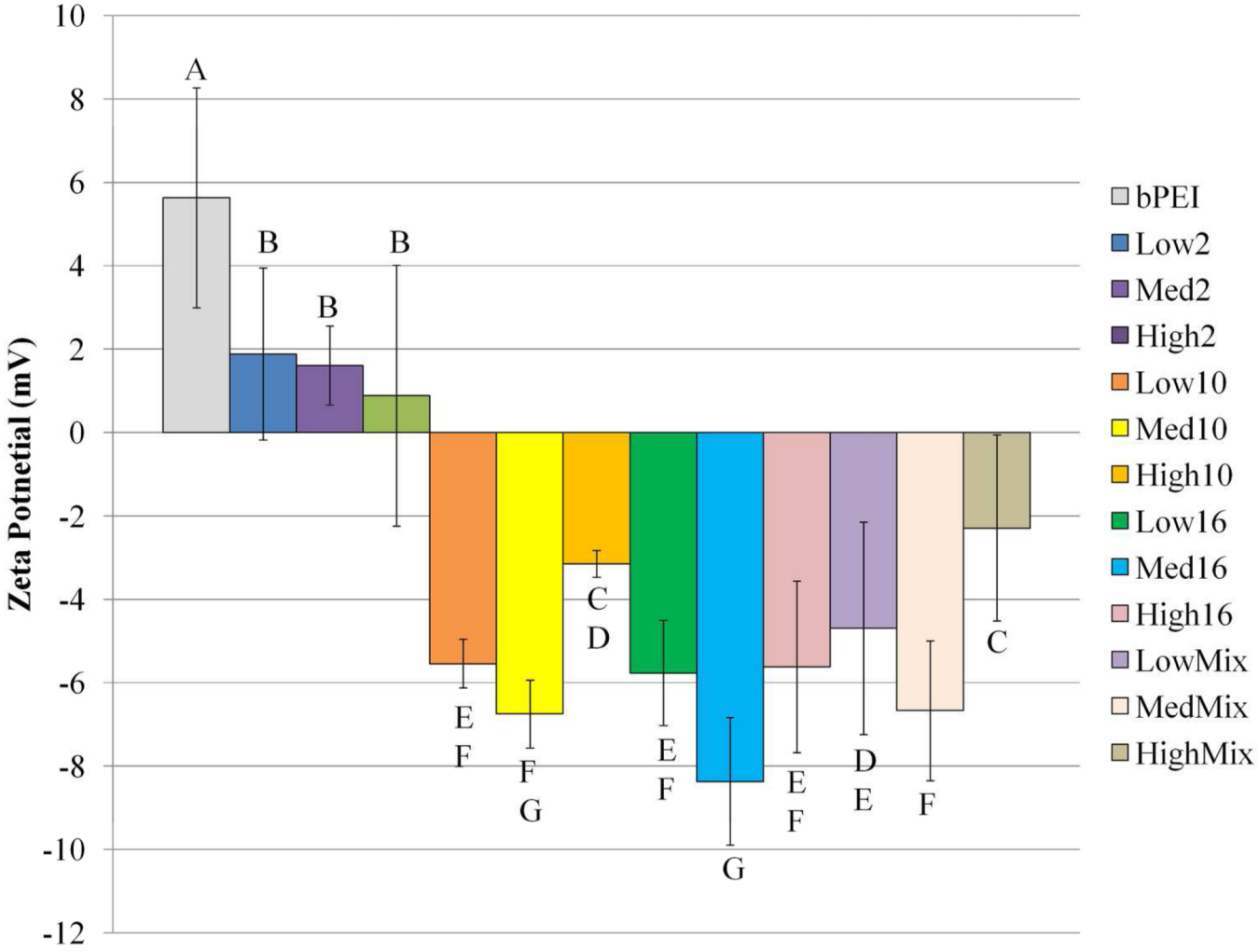

Figure 5.

Zeta potential for each test group of bPEI-HA. Error bars represent 1 standard deviation with n=10, and groups that share letters are not statistically different ($p<0.05$).

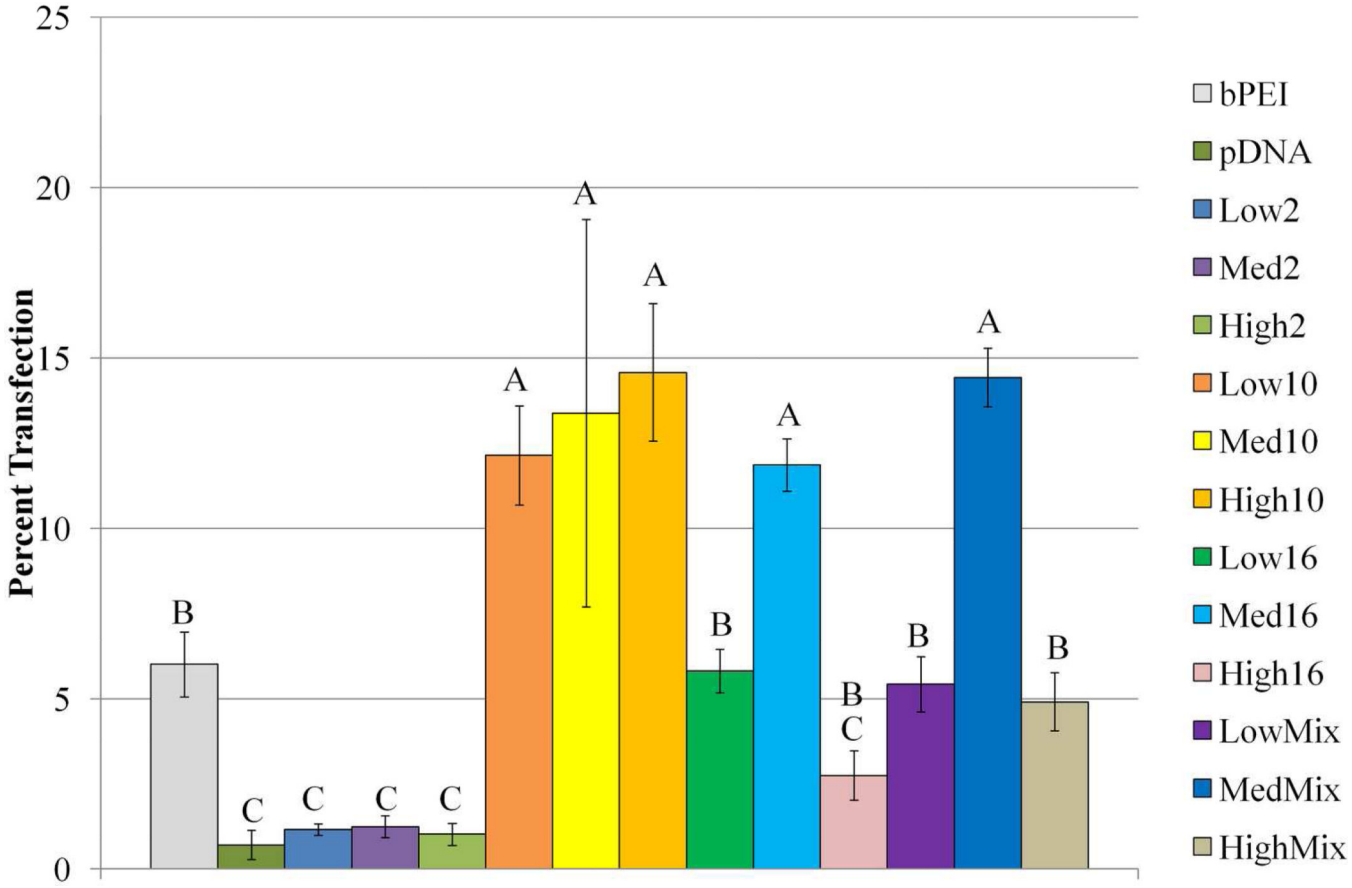

Figure 6.
Transfection efficiency of each group of bPEI-HA, bPEI, or pDNA only on hMSCs. Error bars represent 1 standard deviation with n=6, and groups that share letters are not significantly different ($p<0.05$).

Table 1

Description of the bPEI-HA synthesis groups with respect to HA oligosaccharide type, molar incorporation, and NMR integration ratio.

Group Name	Oligosaccharide Length (# of Saccharides)	Moles of HA per Mole of bPEI	Measured NMR Ratio
Low2	2	4.4×10^{-4}	0.0285
Med2	2	8.8×10^{-4}	0.0640
High2	2	1.3×10^{-3}	0.1049
Low10	10	4.4×10^{-4}	0.1661
Med10	10	8.8×10^{-4}	0.3831
High10	10	1.3×10^{-3}	0.6493
Low16	16	4.4×10^{-4}	0.2666
Med16	16	8.8×10^{-4}	0.6402
High16	16	1.3×10^{-3}	1.0202
LowMix	2, 10, and 16 in a 1:1:1 Molar Mixture	4.4×10^{-4}	0.2074
MedMix	2, 10, and 16 in a 1:1:1 Molar Mixture	8.8×10^{-4}	0.3472
HighMix	2, 10, and 16 in a 1:1:1 Molar Mixture	1.3×10^{-3}	0.5785

Table 2

Complex size as measured by DLS for each test group of bPEI-HA. Results are presented as mean ± standard deviation.

Test Group	Average Complex Size (nm)
Low2	380.7 ± 84.2
Med2	696.6 ± 325.4
High2	341.7 ± 62.4
Low10	231.6 ± 78.4
Med10	340.8 ± 35.0
High10	283.2 ± 325.3
Low16	185.2 ± 2.6
Med16	195.6 ± 35.9
High16	172.2 ± 63.0
LowMix	234.1 ± 146.7
MedMix	337.3 ± 202.3
HighMix	143.0 ± 24.4
bPEI	911.2 ± 313.1