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RESEARCH PAPER

# Modulation of Polyplex Release from Biodegradable Microparticles through Poly(ethylenimine) Modification and Varying Loading Concentration

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## ABSTRACT

***Purpose*** This work investigates the effects of hyaluronic acid (HA) conjugated onto branched poly(ethylenimine) (bPEI) and varying loading concentrations of these polymers complexed with DNA on their release from poly(DL-lactic-co-glycolic acid) (PLGA) microparticles and the transfection of target cells.

***Methods*** To examine the effect of alteration of the gene delivery polymer on the system, we observed the morphology, size, loading efficiency, polymer and DNA release, and the transfection efficiency for the microparticles formed with three internal phase loading concentrations during microparticle formation.

***Results*** Addition of HA to this vector allowed for increased loading concentration within these systems and significantly altered release kinetics without changing the morphology of the particles. The incorporation of HA onto the bPEI backbone significantly increased the transfection efficiency of the complexes released from the corresponding microparticle formulation.

***Conclusions*** The results show that the modification of bPEI with HA and the concentration of loaded polymer/DNA complexes can significantly alter the entrapment and release profiles from PLGA microparticles. This is significant in that it offers insight into the effects of modification of gene delivery vectors on a controlled release system designed to achieve a sustained therapeutic response.

**KEY WORDS** bPEI-HA · controlled release · hyaluronic acid oligosaccharides · non-viral gene delivery · PLGA microparticles

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## INTRODUCTION

Polymeric gene delivery is a growing and promising area of biomedical research because it allows the direct alteration of protein expression within native or introduced cells to achieve a desired therapeutic response (1). By inserting nucleic acids into the cell directly, protein and other nucleic acid targets which might otherwise be impossible to utilize can be used as therapeutic agents or vaccines (2–4). While there are many benefits to utilizing this approach, one aspect that could be considered a drawback is the transient response seen with the application of the non-viral DNA complexes, due to the DNA's non-incorporation into the host cell genome (1). There are many applications in which a more prolonged expression is desirable and necessary, such as the prolonged exposure to a transcription factor to more effectively influence progenitor cell differentiation. For such cases requiring a more sustained expression, controlled release that allows for sustained transfection with DNA complexes over an extended period of time is one potential solution.

One polymeric gene delivery agent which has been thoroughly investigated is branched poly(ethylenimine) (bPEI). While this polymer has been shown to be a relatively effective polymer for gene delivery, it also possesses certain negative characteristics which limit its potential use, especially cytotoxicity (5–7). Alterations to this polymer by pairing hyaluronic acid (HA) with bPEI, especially by forming bPEI-HA through covalently linking the two, have been shown to decrease the cytotoxicity of the resulting polymer while significantly increasing the transfection efficiency (8–13). In this conjugate polymer, the negative charges within the HA mitigate the positive charges associated with bPEI to decrease cytotoxicity. Further, incorporation of specific oligosaccharides of HA can increase transfection efficiency due to intramolecular interactions within the complexes, as well as by potentially allowing association with the hyaladherins on the cell surface (10).

While extensive work has been invested in utilizing poly(DL-lactic-co-glycolic acid) (PLGA)/cationic polymers as a complexation agent for vaccination and siRNA delivery (14,15), this work will focus on the use of PLGA microparticles as a controlled release source for polymer/plasmid DNA complexes. Numerous studies have explored the release of bPEI/nucleotide complexes from PLGA microparticles (2,3,16–23). These studies have investigated the effect of bPEI's inclusion in systems containing nucleotides ranging from siRNA (18,20,24) to plasmid DNA (2,3,21–23,25). However, limited studies have examined the effect of alterations to the bPEI structure itself on the system other than studying the differences between branched and linear PEI and copolymerization with the polymers of the microparticles (25). This work was motivated by the need to understand how alterations within polymers used for gene delivery affect their release from microparticles, which will facilitate more effective control over the design of these polymers for efficient loading and delivery to target cells. This angle of research is especially important when polymers designed for cell targeting are being evaluated.

The studies presented in this manuscript seek to understand how alteration of bPEI with HA oligosaccharides affects the characteristics of PLGA microparticle systems encapsulating these polymers complexed with plasmid DNA. Specifically, this work seeks to compare the morphology, entrapment efficiency, release, and transfection efficiency of PLGA microparticles containing different concentrations of DNA only, bPEI/DNA complexes, and bPEI-HA/DNA complexes. By understanding how the incorporation of HA into bPEI affects controlled release, general conclusions on the use of other alterations to the bPEI system in PLGA microparticles can be elucidated and better understood.

## MATERIALS AND METHODS

### Materials

Sodium borate, sodium chloride, and sodium cyanoborohydrate used for the synthesis and purification of bPEI-HA were purchased from Sigma-Aldrich (St. Louis, MO). HA was purchased from LifeCore Biomedical (Chaska, MN). VivaSpin centrifuge dialysis membranes of 30,000 molecular weight cutoff (MWCO) were purchased from the Sartorius Corporation (Edgewood, NY) and anion exchange columns were bought from GE Lifesciences (Piscataway, NJ). Poly(DL-lactic-co-glycolic acid) of 50-50 copolymer ratio was purchased from Lakeshore Biomaterials (Birmingham, AL). Branched PEI ($M_W$=25,000) was obtained from Sigma-Aldrich (St. Louis, MO). All cell culture was performed with CRL-1764 (ATCC, Manassas, VA) rat fibroblast cells. The cell culture materials: α-MEM, glutamine, trypsin, and phosphate buffered saline (PBS), 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).

### Synthesis of bPEI-HA

Synthesis of bPEI-HA was achieved utilizing a previously described reductive amination reaction (10,13). Briefly, HA and bPEI were added in a 2:1 w:w ratio 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 a temperature of 42°C for 120 h to allow the reaction to fully complete. The resulting product was dialyzed with deionized water three times in a VivaSpin centrifuge dialysis tube with a 30,000 MWCO, according to the manufacturer's protocol, 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 reaction product, $^1$H NMR was performed at room temperature in a Bruker 400 MHz NMR with deuterated water as a solvent and internal reference (δ=4.79). All NMR spectra were processed using MestRe-C software according to a previously established protocol (10).

### Assembly of Polymer/DNA Complexes

In order to correctly track the release of bPEI and bPEI-HA, each was separately tagged with a rhodamine tagging kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions to allow fluorescent detection upon release as previously reported (18). The product was then dialyzed against an excess of ultrapure (type 1) (Super-Q Water Purification System, EMD Millipore, Billerica, MA) water for 72 h, lyophilized, and its mass was quantified on a scale. To form the polymer/DNA complexes, procedures outlined previously were followed (10,13). Briefly, rhodamine tagged bPEI-HA or bPEI (r-bPEI-HA and r-bPEI respectively) was dissolved in PBS at a concentration of 5 mg/ml and 2 mg/ml respectively and filtered through a 0.2 μm filter for sterilization. This solution was then brought to a temperature of 37°C and allowed to sit overnight to dissolve. Once dissolved, each polymer was frozen until use in complex formation. Throughout this and subsequent processes, precautions were taken to avoid rhodamine quenching by light.

To form the internal aqueous phase for microparticle formation, the DNA needed for each synthesis was added to a water solution large enough such that the final volume of the internal phase after addition of the polymer would be a constant 60 μl in every group. Then, tagged bPEI or bPEI-HA polymer solution at room temperature was added dropwise to the prepared DNA solution such that an N:P

ratio was maintained at 7.5:1 for each group. Once mixed, the samples were immediately vortexed, briefly centrifuged, and incubated for 30 min at room temperature to allow for complete complexation. The final solution was then used for microparticle loading or release analysis.

## Microparticle Preparation

Microparticles were prepared using a 5% w/w blend of poly(ethylene glycol) (PEG) in PLGA. PEG was incorporated in these particles to provide molecular scale pores to aid in release (26). This blend was dissolved in dichloromethane at a concentration of 250 mg/ml and microparticles were created using a water-in-oil-in-water double emulsion technique as previously described (27,28). Briefly, the bPEI-HA/DNA complexes were formed as described above for loading into the microparticles. For all groups the internal phase was kept to a constant 60 μl. After incubation at room temperature for 30 min to allow for complete complexation, this 60 μl internal phase was added to the dissolved PLGA/PEG blend and vigorously vortexed for 1 min to form an emulsion. 2.5 ml of 0.3% PVA solution was then added to the emulsion and vortexed for 1 min. Once complete, the resulting solution was added to 200 ml of 1% isopropyl alcohol, 0.15% PVA solution stirring at 800 rpm. The microparticles were allowed to stir for 4 h to allow for complete removal of the dichloromethane from the particles.

After the 4 h solvent evaporation period was complete, the microparticles were removed from the stir plate and strained through a 300 μm mesh to remove larger particles. Once strained, the particles were centrifuged and rinsed three times with ultrapure (type 1) water (Super-Q Water Purification System, EMD Millipore, Billerica, MA) to remove any remaining components of the external phase. Once washed, the microparticles were lyophilized for at least 24 h to completely dry them. Upon removal from the lyophilizer, all samples were purged with nitrogen and stored in a freezer at -20°C until further use.

## Experimental Microparticle Groups

The objective of the studies presented here was to determine the effect of HA conjugation to bPEI on a PLGA microparticle system loaded with polymer/DNA complexes in terms of microparticle morphology, DNA and polymer release, and transfection efficiency. To completely explore the differences in this system between DNA only, bPEI/DNA, and bPEI-HA/DNA complex containing microparticles, three concentrations of internal loading phase DNA were studied for each group; 0.25, 0.75, and 1.25 mg/ml. In groups containing polymers, a constant N:P ratio of 7.5:1 was maintained. This resulted in nine study groups (DNA Low, DNA Middle, DNA High, bPEI Low, bPEI Middle, bPEI High, bPEI-HA Low, bPEI-HA Middle, and bPEI-HA High) that allowed for complete analysis of the effects of loading amount and type on entrapment efficiency, release, and particle morphology to be examined.

## Microparticle Characterization

Microparticle morphology was observed utilizing scanning electron microscopy (SEM) and size was determined through the use of a Multisizer3 Coulter Counter (Beckman Coulter,

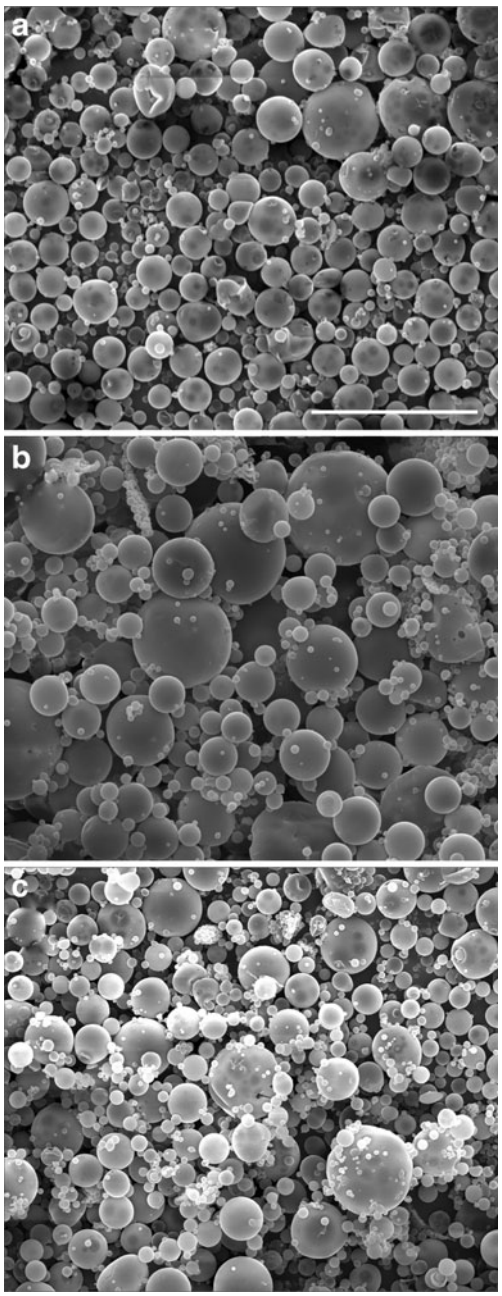


**Fig. 1** Representative SEM images illustrating morphology of particles from (**a**) DNA Middle, (**b**) bPEI Middle, and (**c**) bPEI-HA Middle test formulations. Scale bar in lower right corner of (**a**) indicates 100 $\mu$m and applies to all panels.

Brea, CA) with three samples of 2,500 particles each. For SEM analysis all samples were coated with 20 nm of gold on a Denton Desk V Sputter system (Denton Vacuum, Moorestown, NJ) and examined under a 30 kV beam on a FEI Quanta 400 ESEM FEG (FEI, Hillsboro, OR).

### Entrapment Efficiency

To discover the relative entrapment of bPEI, bPEI-HA, and DNA at each loading concentration, each component was examined individually within the same batch of microparticles. Briefly, 15 mg from each microparticle preparation group was dissolved in 0.5 ml of dichloromethane for 30 min. Once dissolved, the entrapped polymer and DNA were extracted with 1 ml of nuclease free TE buffer as previously described (18) through vortexing for 1 min and centrifuging at 11,000×*g* for 1 min to completely separate the oil and water layers. Once the extraction was complete, r-bPEI and r-bPEI-HA were fluorescently detected using a plate reader while DNA was detected as described below.

### Polyplex Dissociation and Detection

Solution containing polyplexes of polymer and DNA were analyzed utilizing an adaptation of a previously described method of complex dissociation for DNA detection (29). Briefly, aliquots of sample were thawed if necessary and vortexed before use in the assay. Once agitated, 80 μl of sample was added to the wells of an opaque 96 well plate. Each sample was run in triplicate and fresh standards consisting of freshly prepared DNA and polymer (corresponding to the polymer used for each sample's release) were prepared at an N:P ratio of 7.5:1 to be used as a control for each plate. Once prepared, samples were analyzed for rhodamine concentration in a plate reader with excitation/emission wavelengths of 530/575. Once the rhodamine, and thus polymer concentration, was recorded, 160 μl of TE buffer at a pH of 12 and containing 0.5 v/v% PicoGreen dye were added to each sample well. The plate was incubated for 5 min on a shaker table at 60 rpm and then immediately analyzed in a plate reader with excitation/emission wavelengths of 485/530. This analysis was used because it enabled direct correlation between polymer detected and DNA detected for each sample and was used in all cases, except for DNA only release. For groups incorporating DNA only, a PicoGreen assay was performed according to the manufacturer's protocol as polyplex dissociation was not necessary.

### *In Vitro* Release

*In vitro* release was performed in nuclease free PBS. Three samples of 20 mg of microparticles from each group were measured and placed in 1.5 ml nuclease free centrifuge tubes. 0.5 ml of nuclease free PBS were then added to the centrifuge tubes and they were placed on a shaker table at 90 rpm in a warm room set at 37°C. At 6, 12, and 24 h and 2, 3, 4, 7, 11, 14, 18, 21, 25, and 28 days, the particles were centrifuged at 2,000×*g* for 2 min and the supernatant was collected. Following collection, new PBS was added, the microparticles were resuspended, and the samples were returned to the shaker table. Release samples were then stored at −20°C until the release solutions were analyzed as described in the polyplex dissociation and detection section. Release results were broken into four distinct phases for analysis and interpretation: 0–24 h, 1–3 days, 3–18 days, and 18–28 days.

### Transfection Efficiency

The released material from each test group was assessed for its capability to achieve effective transfection. To prepare solutions for analysis, dry microparticles were suspended in PBS at a concentration of 50 mg/ml. These samples were placed on a shaker table at 90 rpm in a warm room held at 37°C. After 1, 2, 3, and 4 weeks, the supernatant was removed from each group

**Table I** Size and Entrapment Efficiency of DNA and Polymer for All Test Groups

| Experimental group | Size (μm) | DNA entrapment efficiency (% of loaded) | Transfection polymer entrapment efficiency (% of loaded) |
|---|---|---|---|
| DNA Low | 15.8 ± 11.1 | $65.5 \pm 7.1^{A}$ | – |
| DNA Middle | 16.6 ± 10.3 | $58.0 \pm 2.4^{A,B}$ | – |
| DNA High | 15.0 ± 9.6 | $43.3 \pm 9.1^{B,C}$ | – |
| bPEI Low | 18.3 ± 12.9 | $25.5 \pm 6.7^{C}$ | $45.2 \pm 8.7^{F}$ |
| bPEI Middle | 12.8 ± 9.8 | $28.9 \pm 2.5^{C}$ | $24.6 \pm 8.2^{G}$ |
| bPEI High | 17.9 ± 14.6 | $2.5 \pm 4.4^{D}$ | $2.7 \pm 1.0^{H}$ |
| bPEI-HA Low | 13.8 ± 9.4 | $61.8 \pm 11.6^{A,B}$ | $63.7 \pm 2.1^{E}$ |
| bPEI-HA Middle | 13.9 ± 9.3 | $61.8 \pm 0.7^{A,B}$ | $61.4 \pm 11.2^{E,F}$ |
| bPEI-HA High | 14.5 ± 9.7 | $50.7 \pm 10.2^{A,B}$ | $43.2 \pm 3.4^{F}$ |
| Blank | 15.8 ± 9.1 | – | – |

Microparticles prepared by drying at room temperature at 800 rpm in a 400 ml beaker for 4 h. Groups containing the same letter are not significantly different from each other with the letters A–D corresponding to DNA entrapment analysis only and E–H corresponding to Polymer Entrapment analysis only. All data are presented as average ± standard deviation for n = 3.

**Table II** DNA Release Rates Presented as Percent of Encapsulated Material Released per Day

| Experimental group | Phase 1 (0–24 h) (% of total encapsulated per day) | Phase 2 (1–3 days) (% of total encapsulated per day) | Phase 3 (3–18 days) (% of total encapsulated per day) | Phase 4 (18–28 days) (% of total encapsulated per day) |
|---|---|---|---|---|
| DNA Low | $14.6 \pm 2.4\%^{A,B}$ | $1.8 \pm 0.0\%^{A}$ | $0.6 \pm 0.1\%^{A}$ | $0.3 \pm 0.1\%^{A}$ |
| DNA Middle | $14.3 \pm 0.6\%^{A,B}$ | $1.7 \pm 0.1\%^{A}$ | $0.7 \pm 0.0\%^{A}$ | $0.1 \pm 0.0\%^{B}$ |
| DNA High | $19.8 \pm 7.7\%^{A}$ | $1.8 \pm 0.7\%^{A}$ | $0.9 \pm 0.2\%^{A}$ | $0.2 \pm 0.0\%^{B}$ |
| bPEI Low | $6.9 \pm 1.8\%^{B,C}$ | $1.9 \pm 0.5\%^{A}$ | $0.7 \pm 0.2\%^{A}$ | $0.7 \pm 0.1\%^{C}$ |
| bPEI Middle | $2.3 \pm 0.7\%^{C}$ | $0.6 \pm 0.1\%^{B}$ | $0.2 \pm 0.0\%^{B}$ | $0.2 \pm 0.0\%^{B}$ |
| bPEI-HA Low | $8.3 \pm 2.1\%^{B,C}$ | $0.2 \pm 0.1\%^{B}$ | $1.5 \pm 0.1\%^{C}$ | $0.2 \pm 0.0\%^{B}$ |
| bPEI-HA Middle | $7.0 \pm 2.9\%^{B,C}$ | $1.1 \pm 0.7\%^{A,B}$ | $1.4 \pm 0.2\%^{C}$ | $0.1 \pm 0.0\%^{B}$ |
| bPEI-HA High | $6.3 \pm 2.4\%^{B,C}$ | $1.2 \pm 0.5\%^{A,B}$ | $2.1 \pm 0.2\%^{D}$ | $0.1 \pm 0.0\%^{B}$ |

Groups with the same letter are not significantly different within each phase. The bPEI High group was excluded from consideration in this experiment due to aggregation during particle formation. Each data point is presented as average ± standard deviation for $n = 3$

and immediately used for transfection. All release samples where handled such that sterility of the samples was maintained.

CRL1764 rat fibroblast cells were seeded onto 12 well plates at a concentration of 20,000 cells per well. After 12 h for attachment, 0.2 ml of release supernatant (corresponding to the release from 10 mg/ml of microparticles) were added to each well with 0.3 ml of serum free media. After 12 h of exposure, 0.5 ml of complete medium were added to each well. 72 h after initial supernatant addition, cells were lifted with 0.05% trypsin and fixed in formalin. The fixed cells were then run through a flow cytometer (Becton Dickinson FACS Scan, Franklin Lakes, NJ) under high flow rate using the CellQuest Pro software from BD Biosciences to assess transfection efficiency. Controls corresponding to fluorescence of 1% of cells treated with supernatant from blank PLGA microparticles were first run through the cytometer to set the criteria to determine transfection efficiency. Finally, a detection limit of 2,000 cells was set for each group in the cytometer.

### Statistics

Statistical analysis was performed on the data collected for microparticle size, encapsulation efficiency, release characteristics, and transfection efficiency using two-way ANOVA with a $p$ value $< 0.05$. Post hoc analysis was performed via Tukey-Kramer HSD to identify statistical significance ($p < 0.05$) between each of the groups. All data are presented as mean ± standard deviation and number of replicates is noted in each case.

## RESULTS

### Microparticle Characterization

Each of the microparticle formulations had a diameter between 12.8 and 18.3 μm and no statistical significance between any of the groups was identified. Particle morphology was investigated with SEM and no significant differences were observed between each particle formulation. Representative SEM images for the medium loading concentration for each case are shown in Fig. 1. Entrapment efficiency was quantified for each of the experimental groups and is shown in Table I. The highest DNA entrapment was found in the DNA only low group with an entrapment of 65.5 ± 7.1%, while the lowest entrapment was in the bPEI High loading group, with a value

**Table III** Polymer Release Rates Presented as Percent Released per Day

| Experimental group | Phase 1 (0–24 h) (% of total encapsulated per day) | Phase 2 (1–3 days) (% of total encapsulated per day) | Phase 3 (3–18 days) (% of total encapsulated per day) | Phase 4 (18–28 days) (% of total encapsulated per day) |
|---|---|---|---|---|
| bPEI Low | $15.3 \pm 3.1\%^{A}$ | $4.9 \pm 1.4\%^{A,B}$ | $1.8 \pm 0.2\%^{A}$ | $1.6 \pm 0.2\%^{A}$ |
| bPEI Middle | $9.4 \pm 5.4\%^{A}$ | $2.7 \pm 1.1\%^{B}$ | $1.6 \pm 0.3\%^{A}$ | $1.4 \pm 0.4\%^{A,B}$ |
| bPEI-HA Low | $43.7 \pm 9.4\%^{B}$ | $8.2 \pm 3.8\%^{A}$ | $0.9 \pm 0.2\%^{B}$ | $0.7 \pm 0.4\%^{B}$ |
| bPEI-HA Middle | $20.8 \pm 4.2\%^{A}$ | $5.9 \pm 1.1\%^{A,B}$ | $0.9 \pm 0.1\%^{B}$ | $0.7 \pm 0.1\%^{B}$ |
| bPEI-HA High | $14.1 \pm 4.8\%^{A}$ | $3.7 \pm 0.7\%^{A,B}$ | $0.9 \pm 0.3\%^{B}$ | $0.7 \pm 0.2\%^{B}$ |

Groups with the same letter are not significantly different within each phase. The bPEI High group was excluded from consideration in this experiment due to aggregation during particle formation. Each data point is presented as average ± standard deviation for $n = 3$

of 2.5 ± 4.4%. This same group also had the lowest polymer entrapment, with 2.7 ± 1.0%, while the bPEI-HA Low group had the highest entrapment with 63.7 ± 2.1%.

Some statistical differences were observed within the nine groups in terms of entrapment efficiency. The DNA and polymer entrapment can each separately be broken into four groups, marked A–D in Table I, within which test formulations in each group were not statistically significant from each other. Specifically, as shown in Table I, the DNA entrapment of groups with unmodified bPEI (bPEI Low, bPEI Middle, bPEI High) was significantly lower than all other groups. There was no statistical significance between the DNA entrapment of any of the bPEI-HA groups (bPEI-HA Low, bPEI-HA Middle, bPEI-HA High), but these values were all significantly higher than the loading of the bPEI complex groups (bPEI Low, bPEI Middle, bPEI High).

In terms of polymer loading, the bPEI Middle and High loading groups were significantly different from all other

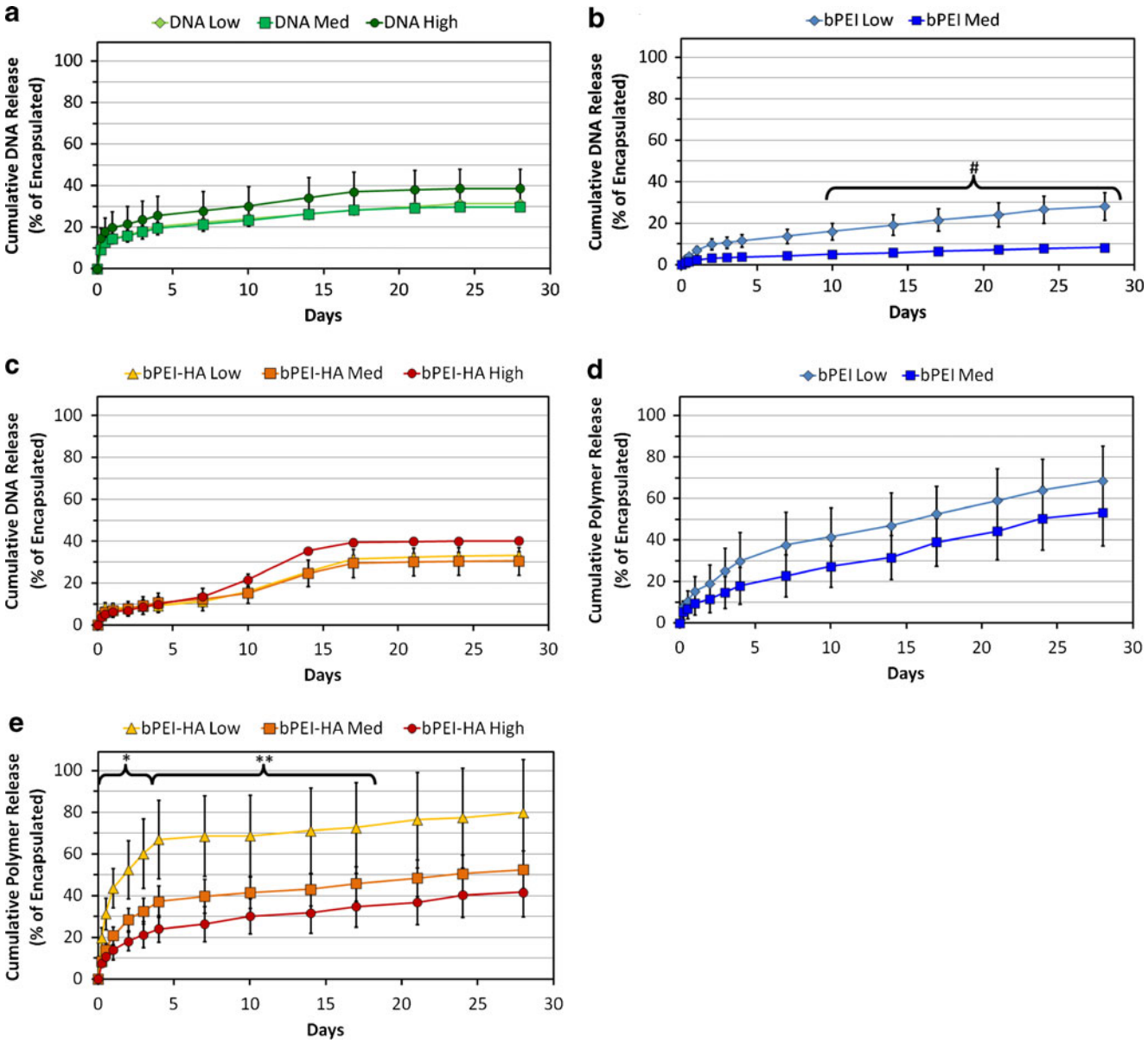


**Fig. 2** DNA release curves from (**a**) DNA only incorporating groups, (**b**) bPEI/DNA complex incorporating groups, and (**c**) bPEI-HA/DNA complex incorporating groups; and (**d**) bPEI release curves from bPEI/DNA complex incorporating groups, and (**e**) bPEI-HA release curves from bPEI-HA/DNA complex incorporating groups. Region "#" denotes the time in which the bPEI Low and bPEI Middle release are significantly different. Region "*" designates the time in which each of the three polymer release curves were significantly different from each other. Region "**" designates the time in which the bPEI-HA Low release is significantly different from the bPEI-HA High release, but not the bPEI-HA Middle release which is not significantly different from either the bPEI-HA High or bPEI-HA Low groups. Release was measured from 20 mg of microparticles in PBS at 37°C for each group. Each data point is presented as average ± standard deviation for $n = 3$.

groups, including each other. Both of these groups had much lower polymer loading than all other groups, with the polymer loading in the bPEI High groups being the lowest statistically. This extremely low incorporation was likely due to macroscopically observable aggregation of the polymer/DNA complexes to the point that they could not be incorporated into the particles. This led to the exclusion of the bPEI High group from the release and transfection efficiency experiments. Finally, the bPEI-HA high and low loading groups had higher polymer entrapment efficiencies, with a statistically significant difference between bPEI-HA High and Low.

### *In Vitro* Release

Tables II and III demonstrate that significant differences in release rate were more frequent between categories (DNA, bPEI, bPEI-HA) of loading with less variation within each category. This trend is also apparent in the overall cumulative release curves presented in Fig. 2.

Groups only incorporating DNA were characterized by the highest relative DNA burst release with the DNA High group demonstrating significantly higher burst (19.8±7.7% in phase 1 for DNA High) when compared to all other polymer containing groups. This was followed by a relatively high phase 2 (1–3 days) release when compared to other groups with the exception of bPEI Low release (1.9±0.5%). Finally release rates from these DNA groups over the third and fourth phase decreased gradually.

For groups incorporating bPEI/DNA complexes, profuse aggregation during polymer/DNA complex formation resulted in the exclusion of the bPEI High group from investigation. DNA release from these groups was characterized by a significantly lower burst release when compared to the DNA only group followed by a gradual slowing of the release rate through phases 2, 3, and 4. Polymer release from these groups was characterized by a relatively small burst release followed by a linear release over time with the phase 3 and phase 4 release rates being significantly higher than the rates seen in bPEI-HA incorporating groups in most cases.

Finally, bPEI-HA incorporating groups had a DNA release profile that was distinct compared to the other groups. The burst release from these groups was in the middle of release rates. The phase one release was relatively low, but a significantly accelerated release for each of the bPEI-HA containing groups within phase 3 was observed. This was in significant contrast to each of the other groups. The polymer release from the bPEI-HA containing groups, on the other hand, was characterized by a large phase 1 and 2 release followed by a reduced phase 3 and 4 release.

### *In Vitro* Transfection

Transfection was observed with each of the polymer containing groups and is presented in Fig. 3. The most effective transfection was observed in the group containing the highest concentration of bPEI-HA/DNA complexes (bPEI-HA High group) with a value of 31.1±17.6% resulting from cell exposure to material released in the second week. For all groups, transfection was characterized by a small but significant initial transfection sustained over 2 weeks with a gradual decline to negligible transfection with week 4 release material.

## DISCUSSION

Entrapment efficiency has been linked to a number of parameters within these water-in-oil-in-water microparticle-based drug delivery systems, including hydrophilicity of the polymers, surfactant concentrations, internal phase volume, and salt concentrations in the external phase (3,16,21,23). This work suggests that internal phase concentration is a key factor when using a DNA complexation agent in microparticle encapsulations. For the loading agent to be fully incorporated, it must be evenly dispersed and not aggregated inside of the internal phase. This information explains the lower loading of all of the bPEI groups relative to bPEI-HA groups. It has been shown that as the concentration of bPEI increases, so does the propensity for aggregation (30), while bPEI-HA complexes are less likely to aggregate due to the inclusion of the HA which acts to stabilize the polymer. During microparticle preparation, bPEI/DNA complexes in the highest concentration could visually be seen aggregating, while no aggregations were noted in the bPEI-HA groups at any concentration. It is hypothesized that these aggregations in the bPEI complex groups were then not effectively incorporated into the particles, leading to the decreased loading efficiency. In

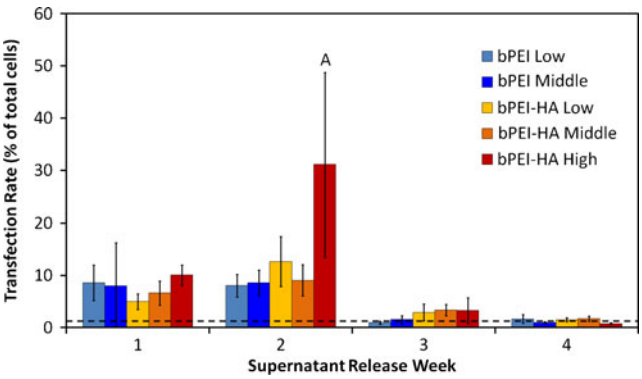


**Fig. 3** Transfection efficiency for each of the polymer containing groups. Cells were exposed to material released over 1 week time periods and tested for GFP expression. Group with "A" is significantly different from all other groups within the same phase ($p < 0.05$). The *black dotted line* corresponds to 1% used as a control value.

fact, the group containing the highest concentration of bPEI (bPEI High) showed negligible encapsulation due to this aggregation effect and was excluded from further investigation. When this group was explored, it was found that the DNA was entrapped in the particles larger than 300 μm which were strained out prior to release studies (data not shown). In other words, the very large aggregates were coated with PLGA. By contrast, the incorporation of HA in the bPEI-HA groups decreased the aggregation of the complexes during loading, resulting in a higher loading efficiency.

The most important factor influencing the release of the entrapped DNA was the polymer type in the polymer-DNA complexes. The incorporation of bPEI-HA into the system significantly accelerated the phase 3 release. This is most probably due to the presence of HA within the system causing accelerated PLGA degradation while also increasing the hydrophilicity of the system. These are two characteristics that have been shown in previous work to accelerate release of plasmid DNA from PLGA microparticles (2,16,22). This accelerated release was observed to a greater degree in the DNA kinetics and not as significantly in the polymer release. While the polymer release kinetics differed between the bPEI and bPEI-HA groups, they maintained the same general release curves with a quick phase 1 and 2 release followed by a slow phase 3 and 4 release. The differences between nucleic acid and bPEI release kinetics have been previously examined using Dynamic Light Scattering, which indicated that the vector and nucleic acid were loaded as complexes, they were released separately and then formed complexes immediately (18,31).

The overall trend in transfection and release kinetics can be explained by examining the degradation rate of the PLGA polymer in each group. Previous work has shown that the DNA not involved with burst release is released according to the PLGA degradation rate found in each of the test groups (22). With this assumption, we can see that the incorporation of an acid, HA, into the system would result in the accelerated degradation of the PLGA system causing an accelerated release of the contained DNA in groups incorporating bPEI-HA.

The transfection efficiency experiments illustrated the potential for released complexes to initiate transfection and result in effective gene expression. For this study, pooled release material was added directly to cells to concentrate the effects of the released material, but it is important to note that if microparticles were added to cells directly, the transfection observed would most likely be distributed over a number of days. Here, the transfection peaked after 2 weeks and then dropped off in each of the groups. The highest transfection was observed in the high concentration of bPEI-HA/DNA complexes after 2 weeks. When taken in context with the DNA release curves and entrapment efficiency, the transfection closely followed the observed trends discussed above. Specifically, as the rate of DNA release dropped precipitously after 2 weeks of incubation, so did the transfection efficiency in all groups. Further, the highest transfection efficiency was observed in the bPEI-HA High group, which had high second week release and high total encapsulation amounts. This suggests that when more DNA and polymer are encapsulated and released in a certain time period, enhanced transfection from bPEI-HA/DNA PLGA microparticles can be achieved.

## CONCLUSIONS

The experiments above illustrate the potential to engineer the release kinetics of polymer/DNA complexes from a PLGA microparticle system while achieving significant transfection efficiency on target cells. Aqueous loading phase concentration is of upmost importance to the loading efficiency. For release characteristics and transfection efficiency, aqueous loading phase concentration does not seem to be as important as the polymer loading type. The addition of HA to the system significantly alters the release by accelerating the sustained release of DNA from the degrading microparticles. These results demonstrate that utilizing a bPEI-HA gene delivery vector in a PLGA microparticle system is a viable and effective method for initiating extended transfection on target cells.

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