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Author(s)	Mahor, Sunil; Dash, Biraja C.; O'Connor, Stephen; Pandit, Abhay
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Mannosylated Polyethyleneimine–Hyaluronan Nanohybrids for Targeted Gene Delivery to Macrophage-Like Cell Lines

Sunil Mahor,[#] Biraja C. Dash,[#] Stephen O'Connor, and Abhay Pandit*

Network of Excellence for Functional Biomaterials, National University of Ireland, IDA Business Park, Galway, Ireland

S *Supporting Information*

ABSTRACT: Nonviral gene delivery systems have a number of limitations including low transfection efficiency, specificity, and cytotoxicity, especially when the target cells are macrophages. To address these issues, the hypothesis tested in this study was that mannose functionalized nanohybrids composed of synthetic and natural polymers will improve transfection efficiency, cell viability, and cell specificity in macrophages. Robust nanohybrids were designed from hyaluronic acid (HA)

and branched polyethyleneimine (bPEI) using carbodiimide chemistry. The reaction product, i.e., branched polyethyleneimine-hyaluronic acid (bPEI-HA) copolymer was subsequently functionalized with mannose at the terminal end of the copolymer to obtain mannosylated-bPEI-HA (Man-bPEI-HA) copolymer. UV spectroscopy and gel retardation studies confirmed the formation of polyplexes at polymer to DNA weight ratio ≥2. Alamar Blue and MTT assay revealed that the cytotoxicity of the developed nanohybrids were significantly ($P < 0.05$) lower than that of unmodified bPEI. Mannose functionalization of these nanohybrids showed specificity for both murine and human macrophage-like cell lines RAW 264.7 and human acute monocytic leukemia cell line (THP1), respectively, with a significant level ($P < 0.05$) of expression of *gaussia* luciferase (GLuc) and green fluorescent reporter plasmids. Internalization studies indicate that a mannose mediated endocytic pathway is responsible for this higher transfection rate. These results suggest that hyaluronan-based mannosylated nanohybrids could be used as efficient carriers for targeted gene delivery to macrophages.
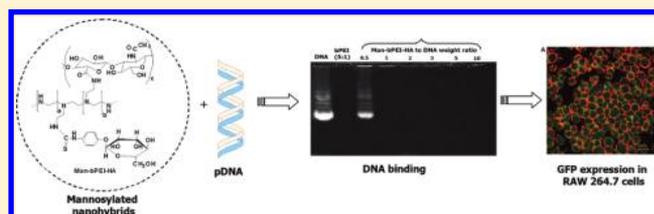
■ INTRODUCTION

In the past two decades, a number of new techniques have been investigated for the transfer of genetic materials into specific cells in order to achieve a therapeutic effect. One of the techniques involves the use of viral vectors because of their higher transfection efficiency; however, toxicity issues such as immunogenicity, inflammation, and oncogenic effects limit their potential clinical translation.[1,2] These limitations have led to the development of nonviral methods of gene transfer, e.g., lipid based and polymeric vectors.[3] Although the lipid based gene transfer methods have been used widely, there are limits in cargo carrying capacity and transfection efficiency. Therefore, the use of polymeric systems has been a focus of recent investigation. Specifically, polyethyleneimines (PEIs) have been extensively investigated for various gene transfer applications.[4,5] The transfection efficiency of PEIs can be attributed to the "proton sponge effect" allowing endosomal or lysosomal escape and transfer of DNA to the nucleus.[1−3] However, the inherent cytotoxicity of PEIs has limited their applications for in vivo gene delivery.[8−10] Furthermore, PEIs are also known to cause complement activation, coagulation of blood cells, and aggregation of serum proteins.[11] Several strategies have been investigated to overcome these limitations. One of the strategies has been to mask the strong cationic group by the use of maltose and dextran,[12] poly(ethylene glycol),[13] and hyaluronic acid.[14]

Hyaluronan or hyaluronic acid (HA) is a widely used biomaterial which is relatively unexplored as a delivery vehicle for gene transfer.[15,16] Hyaluronic acid is the only nonsulfated glycosaminoglycan (GAG) that is abundant in synovial fluid and extracellular matrix (ECM). Different molecular weights of HA have various physiological effects and molecular weight profiles in the range 10^3-10^7 Da have been studied for HA in numerous applications. Recently, hyaluronic acid was reported to mask cytotoxicity of PEI by covalent conjugation.[17]

For effective and successful gene delivery and expression in specific cell types, a selective targeting approach is often desirable.[18,19] To improve gene transfection efficiency and avoid nonspecific interaction between plasma proteins and the cell membrane, receptor-mediated endocytosis of the polymer/DNA complexes is required. Mannose receptors are abundantly expressed on antigen-presenting cells such as macrophages and dendritic cells.[20−22] The introduction of mannose to various nonviral gene delivery carriers such as polylysine,[23] polyethyleneimine,[4,24] cationic liposomes,[25] and chitosan[26] has shown an improved cellular uptake and transfection efficiency in macrophage cells.
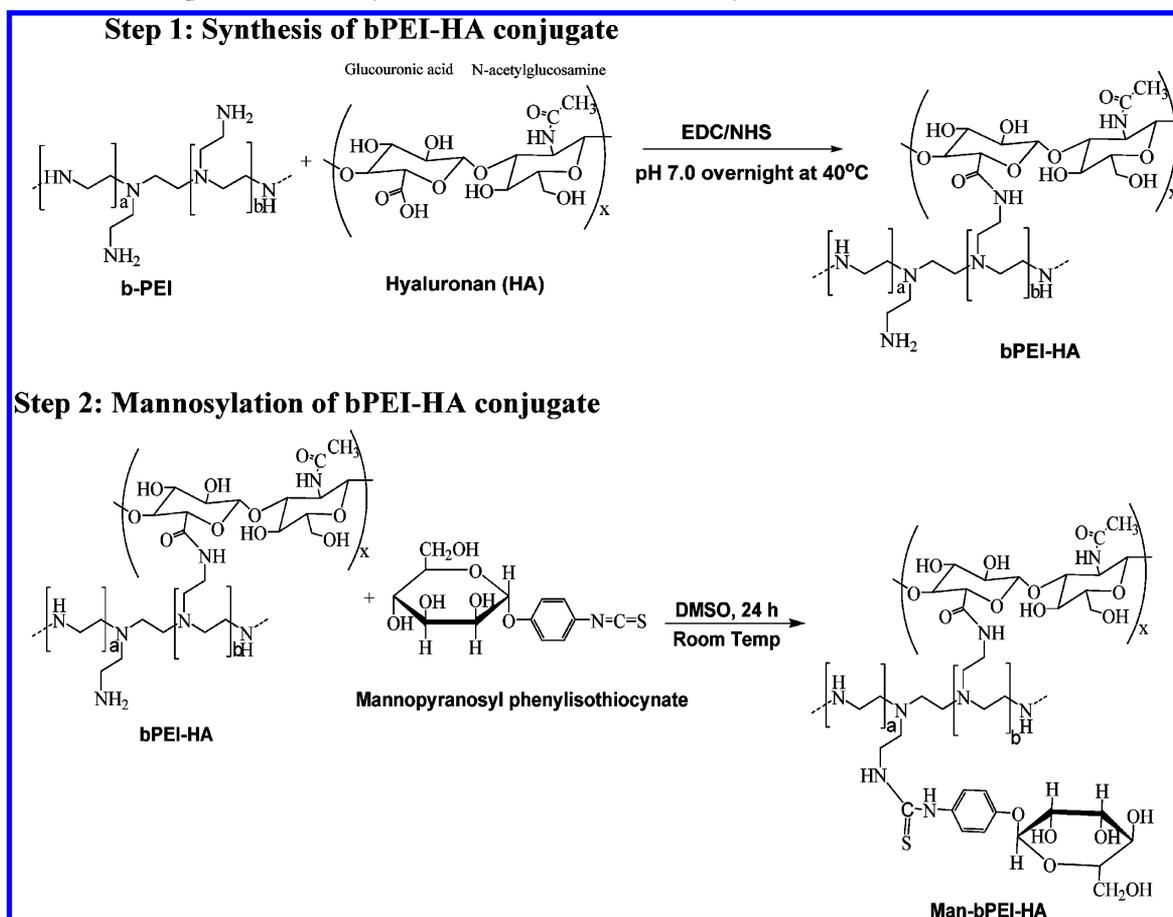
The aim of this study was to synthesize mannose-functionalized nanohybrids based on branched PEI (bPEI)

Scheme 1. Schematic Representation of Synthesis of Man-bPEI-HA Nanohybrids

and HA with the goal of targeting macrophages. The first objective of this study was to reduce the toxicity of bPEI by coupling bPEI to hyaluronan to form a bPEI-HA copolymer using carbodiimide chemistry. The second objective was to modify bPEI-HA into Man-bPEI-HA nanohybrids by tethering mannose to terminal groups of bPEI-HA for increased transfection efficiency through receptor-mediated endocytosis in two different macrophage cells: RAW 264.7 and THP1 from murine and human source, respectively. These objectives were investigated by assessment of the physicochemical properties of Man-bPEI-HA/pDNA complexes by particle size measurement, zeta potential measurement, gel electrophoresis, UV spectroscopic analysis, and pDNA protection studies. The in vitro cytotoxicity, transfection efficiency, and internalization of the Man-bPEI-HA/pDNA complexes were also evaluated with respect to the selective use of these nanohybrids for both murine and human macrophages.

■ EXPERIMENTAL SECTION

Materials. Hyaluronan (M_w 750−1000 kDa) was purchased from CPN Ltd. (Czech Republic). D-Mannopyranosylphenyl isothiocyanate, branched PEI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC), and N-hydroxysuccinimide (NHS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Ireland, and used without any purification. Deionized (DI) water was used throughout the studies unless otherwise stated.

Plasmid Propagation and Isolation. XL1-Blue (Stratagene USA) competent cells were transformed and selected twice in antibiotic containing LB broth and on LB agar plates. Plasmid amplification was carried out using a QIAGEN EndoFree Plasmid Giga Kit (QIAGEN, USA) following the manufacturer's instructions. Plasmid purity was confirmed by UV spectroscopy (NanoDrop ND1000 Spectrophotometer, Thermo Scientific) and gel electrophoresis. Also, in order to avoid recontamination of plasmid DNA after initial endotoxin removal, only new plasticware which is certified to be pyrogen- or endotoxin-free for storage was used. *Gaussia* princeps luciferase plasmid (pCMV-GLuc) was obtained from New England Biolabs UK and its expression was assayed with a kit from the same manufacturer. Plasmid DNA expressing green fluorescent protein (pCMV-GFP) was also amplified and propagated following the same protocol.

Preparation of Man-bPEI-HA Nanohybrids. Man-bPEI-HA conjugates were synthesized in a two-step reaction as shown in Scheme 1. In the first step, HA was conjugated with branched PEI using the method described previously with modifications.[27] Briefly, 25.0 mg of HA (5.0 mg/mL) was dissolved in 0.1 M phosphate buffer and the pH was adjusted to 7.0. N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide and NHS were added in a 20% molar excess based on the carboxyl group and the reaction mixture was stirred overnight at 40 °C. 125.0 mg of bPEI (10 mg/mL) was added to the reaction mixture under continuous stirring at 40 °C. The reaction was allowed to complete by incubation for another 12 h under the same conditions. The conjugates were purified by dialysis for 48 h using a dialysis membrane (MW cutoff 50 kDa Spectrapore) and washed four times with 0.1 M phosphate buffer. Finally, the

buffer was exchanged with distilled water (four washes). The bPEI-HA conjugates were collected and lyophilized (Virtis Advantage 2.0, UK).

In the second step, 100 mg of purified bPEI-HA conjugate obtained from the previous reaction was dissolved in 0.1 M carbonate buffer (pH 9.0). 2.5 mg of D-mannopyranosylphenyl isothiocyanate (15% molar excess) was dissolved in 1 mL of DMSO, and added to the reaction mixture and allowed to react for 24 h at room temperature to complete the reaction. Man-bPEI-HA conjugate was further purified by dialysis for 48 h using a dialysis membrane (MWCO 8 kDa Spectrapore) and washed four times with 0.1 M carbonate buffer (pH 9.0) and then with DI water (four washes). The bPEI-HA conjugates were then collected and lyophilized. FITC labeling was performed as described in a previous study.[28] Briefly, a weight ratio of 1:40 of FITC to bPEI-HA-Man was shaken at 4 °C overnight. The unbound FITC was removed by dialyzing in water for one day.

Fourier Transform Infrared (FTIR) Analysis of Man-bPEI-HA. Purified conjugate samples were prepared by the KBr pellet method and analyzed by FTIR spectroscopy using an FTIR AIM-8800 spectroscope (Shimadzu, Japan) (32 scans, resolution 4.0 and wavenumber range 4000−500 cm^{-1}).

^{1}H Nuclear Magnetic Resonance (^{1}H NMR) Assay. The Man-bPEI-HA nanohybrids were characterized using proton nuclear magnetic resonance (^{1}H NMR). ^{1}H NMR was carried out on a 400 MHz JEOL NMR with *DELTA* processing software. The chemical shifts were referenced to the lock D$_2$O.

Determination of Primary Amines in Man-bPEI-HA by Ninhydrin Assay. Unmodified bPEI, HA-bPEI, and Man-bPEI-HA were characterized for number of primary amine groups and estimated by ninhydrin assay.[29] The standard curve was prepared and plotted using polyamidoamine dendrimer G-4 (Sigma-Aldrich, UK) as a standard. Briefly, 100 μL of polymer solution (0.2 mg/mL) and a standard having different concentrations were added with 100 μL of sodium acetate buffer (pH 5.4) into 1.5 mL microcentrifuge tubes. 100 μL of freshly prepared ninhydrin reagent was added to each centrifuge tube, after which the tube was placed in a boiling water bath for 10 min and cooled to room temperature. After cooling, 300 μL of 50% v/v isopropyl alcohol in water was added to each tube. Finally, the absorbance of each solution was measured at 570 nm in triplicate against a reagent blank by a UV−vis spectrophotometer (VarioskanFlash-4.00.53).

Gel Retardation Studies of Man-bPEI-HA/pDNA Complexes. The binding ability of the Man-bPEI-HA with plasmid DNA was studied by agarose gel electrophoresis as described previously with slight modifications.[30] The Man-bPEI-HA/pDNA complexes containing luciferase plasmid were prepared with different polymer to DNA weight ratios ranging from 1:1 to 10:1 by keeping the plasmid DNA amount constant and varying the amount of Man-bPEI-HA. Thereafter, the complexes were loaded in individual wells of 0.8% (w/v) agarose gel in TAE buffer containing SYBR Safe stain (Invitrogen, USA). The samples were run at 100 V for 90 min, and the gel was then visualized on a UV transilluminator (G-Box, Syngene, UK).

Size and Zeta Potential of Man-bPEI-HA/pDNA Complexes. The particle size and surface charge of the Man-bPEI-HA/pDNA complexes were measured using a Malvern's zetasizer (Nano-ZS90, Malvern Instrument Ltd., UK). Man-bPEI-HA/pDNA complexes at different polymer to pDNA weight ratios from 0.5:1 to 10:1 were prepared in PBS

(pH 7.4) following the same method as used in the previous section. The particle size measurement was performed with a He−Ne laser beam at 658 nm and a scattering angle of 90°. The particle size measurement was repeated for 3 runs for each sample, and the data were reported as the average of the effective mean diameters of 3 runs. The zeta potential measurement was repeated for 3 runs per sample, and the data were calculated automatically using the software from the electrophoretic mobility based on the Smoluchowski's formula. Before starting measurement, the complex solution was subjected to 10 times dilution with PBS (pH 7.4) to mimic the condition employed for in vitro transfection experiment.

Protection Studies of Man-bPEI-HA/pDNA Complexes against DNase I. For DNA digestion assay, the complexes were subjected to 2 μL of DNase I (2 IU to 1 μg of DNA) in DNase I buffer (400 mM Tris-HCl (pH 8.0), 100 mM MgSO$_4$, and 10 mM CaCl$_2$) for 30 min at 37 °C. The reaction was terminated with 4 μL of 0.5 M EDTA (pH 8.0) and then placed on ice for 10 min. The samples were divided in half before running in agarose gel electrophoresis. One-half of the samples were pretreated with a high concentration (10 mg/mL) of polyglutamic acid (PGA) for 30 min at 37 °C.[31] All samples were then subjected to 1% agarose gel electrophoresis stained with SYBR Safe in TAE buffer at 100 V for 60 min. DNA was visualized on a UV transilluminator (G-Box, Syngene, UK).

Cell Culture Experiments. Cellular metabolic activity (alamarBlue reduction, and MTT assay) and transfection activity (G-luciferase activity) of developed nanohybrids were studied using RAW 264.7 and THP1 cells. RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C in humidified 5% CO$_2$. THP1 nonadherent cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% P/S and L-glutamine at 37 °C in humidified 5% CO$_2$. Culture of adherent macrophages from THP1 monocytes were achieved by using phorbolmyristate acetate (PMA) in a differentiation media.[32] Briefly, the THP1 cells, when they reached a density of 800 000−1 000 000 cells/mL, were cultured on tissue culture plates in a RPMI-1640 differentiation media containing 5 g/L glucose, 1% P/S, and L-glutamine and PMA at a final concentration of 100 ng/mL for 24 h.

MTT Assay. The effect of uncomplexed Man-bPEI-HA, bPEI-HA, and unmodified bPEI on RAW 264.7 and THP1 cells metabolic activity was evaluated by MTT test as described previously.[30−33] Briefly, RAW 264.7 and RAW cells were seeded in 96-well microtiter plates at a density of 1×10^4 cells/well. After 24 h, culture medium was replaced with complete medium (DMEM + 10%, FBS + 1% P/S for RAW 264.7 and RPMI-1640 + 10%, FBS + 1% P/S for THP1) with uncomplexed Man-bPEI-HA, bPEI-HA and unmodified bPEI and incubated for two different time points, i.e., 4 h and 24 h. Then, the medium was replaced with MTT solution in PBS and incubated for 2 h at 37 °C in humidified 5% CO$_2$. The unreacted dye was aspirated, formazan crystals were dissolved in 100 μL of DMSO, and absorbance was read at 570 nm with a background correction at 690 nm using a microplate reader (VarioskanFlash-4.00.53). Relative cell growth was related to untreated cells (control) and % cell viability was calculated by $[A_{(test)} \times 100]/A_{(control)}$. Similar protocol of MTT was followed to evaluate the metabolic activity of the complexed DNA of different polymer /pDNA weight ratio (0.1, 1, 2, 3, 5, and 10) on RAW 264.7 and THP1 macrophages. Also, the experimental

conditions were kept similar to those of in vitro transfection experiments mentioned below. They were kept in the incubator for 24 h before performing any experiments on them.

In Vitro Transfection Studies. All transfection studies were performed in triplicate. A total of 5×10^4 RAW 264.7 (specific for mannose receptor) and NIH3T3 (nonspecific for mannose receptor) cells per well were seeded on 48-well plates 24 h prior to transfection. The medium was replaced with the fresh medium (DMEM + 10% FBS + 1% P/S) immediately before transfection. Man-bPEI-HA/DNA complexes were prepared by adding the appropriate amount of Man-bPEI-HA or bPEI-HA in serum free DMEM to pDNA and incubated at room temperature for 30 min under mild agitation. The transfection complexes were appropriately diluted with serum-free DMEM. 50 μL of the complex solution was then added to each well and cells were incubated at 37 °C in 5% CO_2 atmosphere. After 4 h, the medium was replaced with fresh medium (DMEM + 10%, FBS + 1% P/S) and incubated for another 44 h. Cell supernatant was then collected and assayed for GLuc activity using a GLuc assay kit following the manufacturer's protocol. Results were then calculated and expressed as relative light units (RLU) normalized with untreated cells (negative control).

For competition studies, cells were treated with Man-bPEI-HA/pDNA complexes in the presence of mannosylated bovine serum albumin (Man-BSA) (a specific competitor for mannose receptors) and BSA (a nonspecific competitor).[23,34] Man-BSA was prepared using BSA and D-mannopyranosylphenyl isothiocyanate. Briefly, 100 mg BSA was dissolved in 0.1 M carbonate buffer (pH 9.0) and D-mannopyranosylphenyl isothiocyanate of 15% molar excess was dissolved in 1 mL of DMSO, and added to the reaction mixture and allowed to react overnight at room temperature to complete the reaction. Man-BSA conjugate was further purified by dialysis. Briefly, for these specific transfection studies a total of 5×10^4 RAW 264.7 and THP1 cells per well were seeded on 48-well plates 24 h prior to transfection. The medium was replaced with the aforesaid fresh medium immediately before pretreatment of Man-BSA and BSA. Both Man-BSA and BSA were used at a final concentration of 100 nmol and incubated for 30 min before the transfection studies.[35] Man-bPEI-HA/pDNA, bPEI-HA/pDNA, and bPEI/pDNA of different ratio (0.1, 1, 2, 3, 5, and 10) were used for these experiments and a similar protocol, as mentioned above, was followed for the quantification of the transfection efficiency.

pCMV-GFP reporter gene was used to investigate further the in vitro transfection efficiency of the nanohybrids. For these studies, a total of 1×10^5 RAW 264.7 and THP1 cells per well were seeded on 6-well plates 24 h prior to transfection. Then, the cells pretreated with Man-BSA and BSA were incubated with Man-bPEI-HA/pDNA complexes of ratio 2. The cells were mechanically harvested using cell scrapper and washed and resuspended in FACS buffer in order to analyze by FACS.

Fluorescence Microscopy Studies for Reporter Gene GFP Expression. RAW 264.7 and THP1 cells were seeded 24 h prior to transfection studies in a 35 mm MatTek's glass chamber tissue culture slide using 500 μL of complete medium and 10 000 cells/chamber, incubated at 37 °C, 5% CO_2. After 24 h of incubation, the medium was replaced with aforementioned fresh medium and incubated with Man-bPEI-HA/pDNA complexes at optimum polymer to pDNA weight ratio, i.e., 2. After 4 h, the medium was again replaced with fresh medium and cells were incubated for another 44 h at 37 °C, 5%

CO_2. Cells were then washed with cold PBS solution and fixed with formaldehyde solution overnight at 4 °C. Cells were incubated with DAPI (Invitrogen, USA) for cell nucleus staining, mounted with Vectashield, and imaged with OLYMPUS fluorescence microscope.

Colocalization Studies. Man-bPEI-HA labeled with FITC was for the colocalization study. Man-bPEI-HA/pDNA at a ratio of 2 was prepared and incubated with both RAW 264.7 and THP1 cells for 6 h. The cells were pretreated with Man-BSA and BSA for the competitive study. The cells were washed after the required time of incubation in PBS. Lysotracker Blue was used as per manufacturer's instructions to stain the lysosomes in live cells. Cells were kept in the incubator for 2 h. The cells were fixed with 2% paraformaldehyde for 30 min, and this was followed by several washes with PBS. The cells were then observed under the fluorescence microscope (Olympus IX81). Lysotracker Blue staining was tinted as blue and the polyplex as green. The images were then merged to characterize the colocalization.

Statistical Analysis. One-way ANOVA was performed using *GraphPad Prism* v 5.1 to detect significant differences between various groups. Differences were considered significant for $p < 0.05$.

■ RESULTS

Synthesis and Characterization of Man-bPEI-HA Nanohybrids. The suitability of mannosylation in targeting of nanocarriers for macrophages has been explored in the present investigation. Man-bPEI-HA nanohybrids were successfully developed as target specific gene delivery carriers. As shown in Scheme 1, mannosylated nanohybrids of bPEI and HA were prepared by a two-step method. Because bPEI has been used in various gene delivery applications, it was coupled to HA by using water-soluble EDC to alleviate the cytotoxicity of bPEI in the form of bPEI-HA.[36] Man-bPEI-HA nanohybrids were prepared by thiourea reaction between the isothiocyanate group of mannopyranosylphenyl isothiocyanate and the amine groups of bPEI-HA with a lower degree of substitution for targeting both murine and human macrophages (RAW 264.7 and THP1) that have an overexpression of mannose receptors. To improve yields, reaction conditions were optimized for pH, reaction temperature, and time.

FTIR spectroscopy involves the measurement of the wavelength and intensity of absorption of infrared light through the excitation of molecular vibrations, which provides predictive information about representative functional groups and changes in the molecular structure of organic materials. The representative FTIR spectra of the various conjugates and intermediates were studied and are shown in Figure 1. Absorption peaks were observed in the region 3300−3350 cm^{-1} due to the N−H stretching in primary amines (Figure 1A). FTIR studies also revealed the characteristic peak in the region of 1600−1640 cm^{-1} due to the presence of C=O (carbonyl) groups in HA (Figure 1B). bPEI-HA copolymers showed characteristic peaks in the region of 3309 cm^{-1} and 1575.91 cm^{-1} due to N−H stretching and C=O stretching, respectively (Figure 1C). Upon mannosylation, characteristically strong peaks were observed in the region of 1146.35 cm^{-1} and 1550−1620 cm^{-1} due to C=S (thionyl) and C=C stretching, respectively (Figure 1D). The Man-bPEI-HA nanohybrids were characterized for conjugation using ^{1}H NMR (Figure 2). The ^{1}H NMR spectra of bPEI−HA as compared to that of HA and PEI has both a methyl peak of
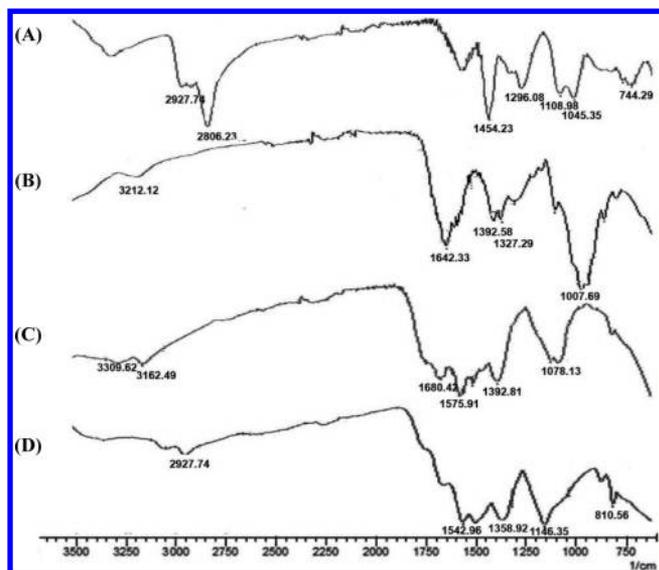
Figure 1. FTIR spectra of (A) branch-type polyethyleneimine (bPEI), (B) hyaluronic acid (HA), (C) bPEI-HA, and (D) Man-bPEI-HA.

acetoamide group of HA at 1.9 ppm and a bPEI peak at 2.5−3.2 ppm shifted from 2.5 ppm as reported in previous literatures (Figure 2A−C).[27] In the final step, the thiourea reaction between the isothiocyanate group of mannopyranosylphenyl isothiocyanate and the amine groups of bPEI-HA forms the nanohybrid Man-bPEI-HA. This is clearly evident in the ^{1}H NMR spectra of Man-bPEI-HA where three peaks corresponding to HA (1.9 ppm), PEI (2.5−3.2 ppm), and a relatively weak signal of mannose (7 ppm) can be seen as reported elsewhere (Figure 2D).[26] We hypothesize that the relatively weak signal of mannose at 7 ppm in ^{1}H NMR spectra can be attributed to the low proportion of mannose in the overall nanohybrid composition.
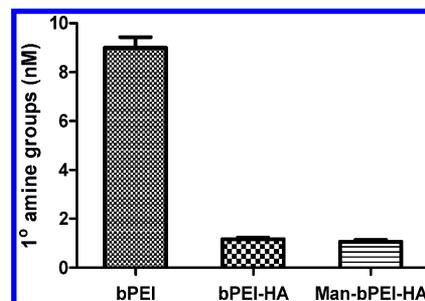
Determination of Primary Amines by Ninhydrin Assay. The ninhydrin assay was performed to quantify the number of primary amines in Man-bPEI-HA and bPEI-HA nanohybrids and compared with unmodified bPEI. The ninhydrin assay revealed that the unmodified bPEI possesses a higher number of primary amines than do bPEI-HA and Man-bPEI-HA nanohybrids and the conjugation of HA with bPEI reduced the number of primary amines in the conjugates (Figure 3). There was an almost comparable level of primary amines in Man-PEI-HA when compared with bPEI-HA. The degree of substitution for mannose residue in Man-HA-PEI was calculated and found to be nearly 8% of amines.

Gel Retardation Studies of Man-bPEI-HA/pDNA Complexes. Gel retardation studies of complexation of pDNA with Man-bPEI-HA conjugates revealed that polymers were able to bind with pDNA with an increase in polymer to pDNA ratio (Figure 4). At a low polymer to pDNA weight ratio such as 0.5:1, pDNA was observed in migrated lane (free pDNA) as well as in retarded form (complexed pDNA). At a polymer to pDNA weight ratio of 1:1 and higher, the pDNA was observed in the gel pocket, most probably due to the complexation of pDNA with polymers. pDNA retention in the gel pocket suggested that the migration ability of plasmid pDNA was retarded due to the polyplex formation.

Size and Zeta Potential Analysis of Man-bPEI-HA/pDNA Complexes. The hydrodynamic diameters of pDNA complexes of mannosylated and nonmannosylated bPEI-HA
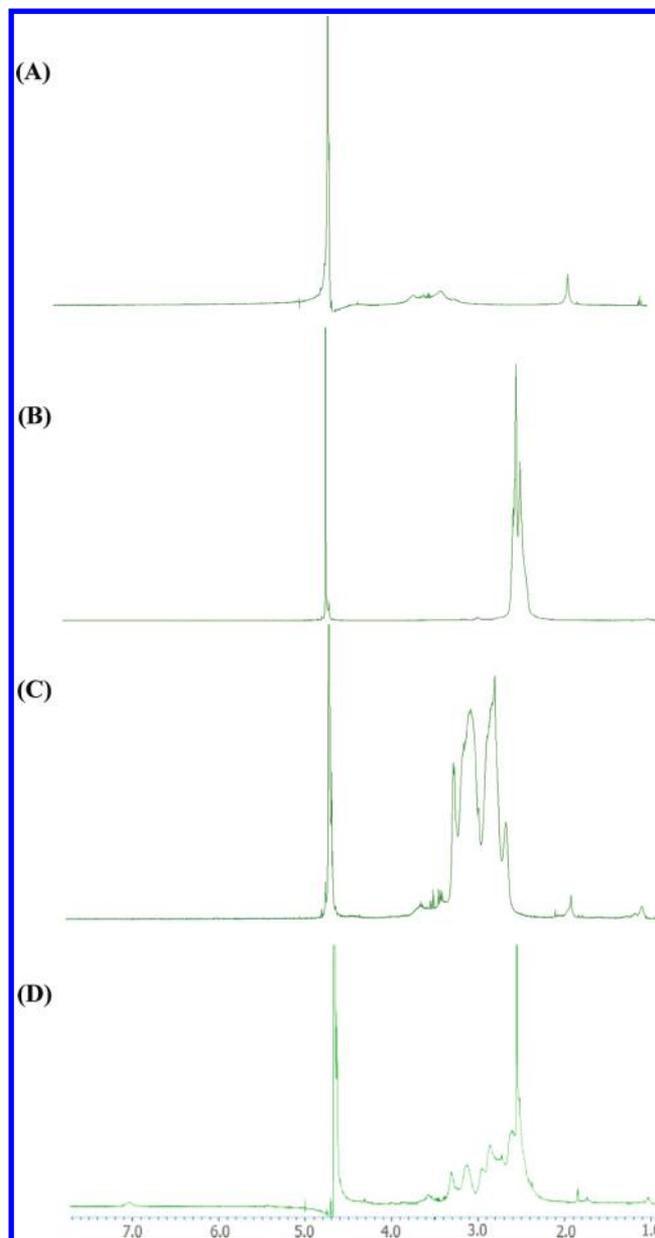
Figure 2. Representative ^{1}H NMR spectra of Man-bPEI-HA. ^{1}H NMR spectra of (A) HA, (B) bPEI, (C) bPEI-HA, conjugate and (D) Man-bPEI-HA nanohybrid.


Figure 3. Physicochemical characteristics of Man-bPEI-HA conjugates. Quantification of primary amines by ninhydrin assay. Man-bPEI-HA, bPEI-HA, and unmodified bPEI were reacted with ninhydrin reagent and primary amino groups were estimated spectrophotometrically. Data are shown as mean ± SD (n = 3).
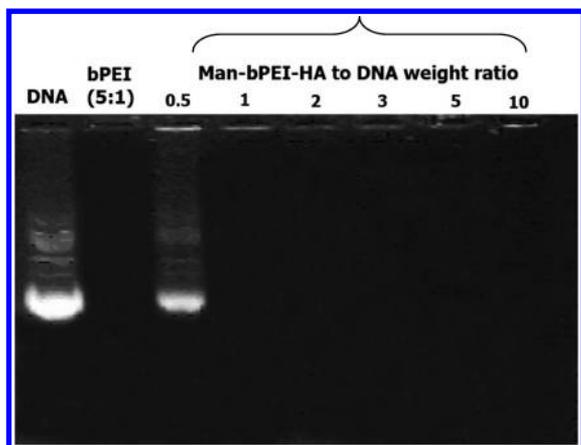
Figure 4. Polymer/pDNAcomplexation studies at different weight ratio of polymer to pDNA using agarose gel electrophoresis analysis.

nanohybrids were dependent upon weight ratio (Figure 5). Man-bPEI-HA/pDNA complexes were examined for size at
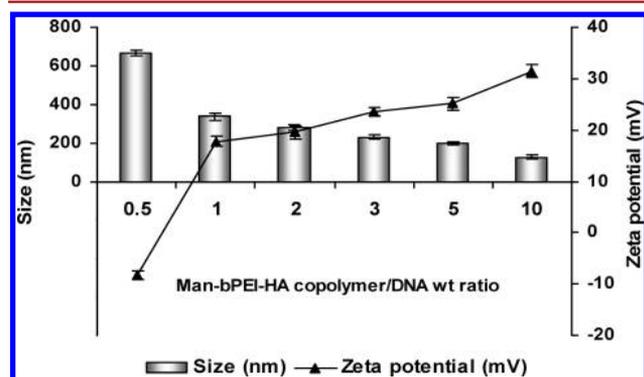
Figure 5. Size and zeta potential analysis of Man-bPEI-HA/pDNA complexes at polymer to pDNA weight ratio. (Data are shown as Mean $\pm$ SD, $n = 3$.)

different weight ratios ranging from 0.5 to 10. The higher polymer to pDNA weight ratios led to a decrease in complex size. A Man-bPEI-HA to pDNA weight ratio of 0.5 caused larger complexes (664.07 ± 13.32 nm) compared to a weight ratio of one (336.43 ± 18.79 nm). Furthermore, this increment of weight ratio indicates more condensation of polyplexes and further reduction in complexes size.

A linear relationship was observed between the zeta potential and Man-bPEI-HA to pDNA weight ratio (Figure 5). A Man-bPEI-HA to pDNA weight ratio of 0.5 showed negative zeta potential of -8.27 ± 0.85 because of incomplete pDNA condensation and complexes bearing more pDNA on their surface, and thereafter showed positive surface charge (17.77 ± 1.06, 19.5 ± 1.21, 23.53 ± 0.97, 25.1 ± 1.18, and 31.47 ± 1.09 for Man-bPEI-HA to pDNA weight ratios of 1, 2, 3, 5, and 10, respectively).

pDNA Protection Studies of Man-bPEI-HA/pDNA Complexes against DNase I. The endonuclease activity of DNase I results in fragmentation of unprotected plasmid DNA. Plasmid DNA complexation with polymers is expected to protect plasmid DNA from enzymatic degradation. In this study, naked pDNA and Man-bPEI-HA/DNA complexes at different polymers to pDNA weight ratios were treated with DNase I and their protection ability was evaluated by agarose

gel electrophoresis (Figure 6A). Naked pDNA was not seen in the lane because of complete degradation within 30 min of
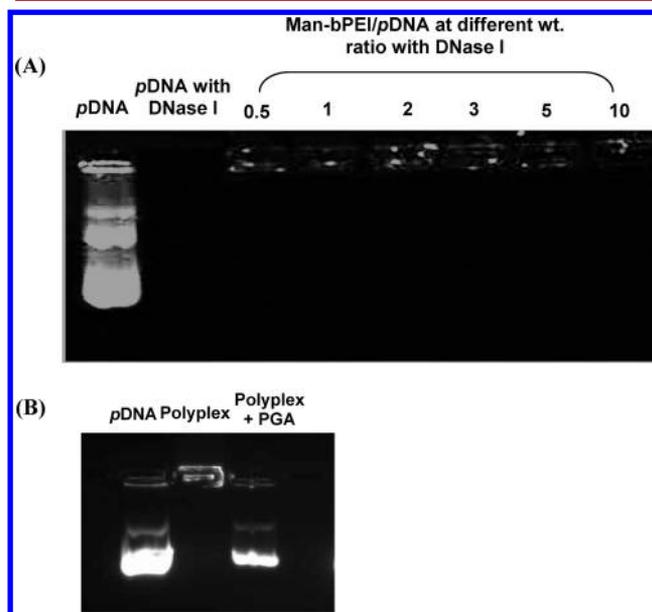
Figure 6. DNA protection studies against DNase I in DNase I buffer. Man-bPEI-HA/pDNA complexes were prepared at different weight ratio of polymer to pDNA and treated with DNase I and reaction were stopped by using 0.5 M EDTA. Samples (A) untreated with polyglutamic acid (PGA) and (B) treated with PGA (polymer/DNA ratio 2) were run on 1% agarose gel electrophoresis and visualized under UV transilluminator.

treatment of DNase I (lane 2), whereas complexed pDNA were detected in the gel pocket. These results illustrated that pDNA retention in the gel pockets, most likely due to the complexation of Man-bPEI-HA with pDNA, protected the pDNA from enzymatic degradation against DNase I. Further investigation of this was carried out by incubating these DNase I treated pDNA complexation with PGA (Figure 6B). The agarose gel electrophoresis in lane 1 showed migrated native pDNA (control), while the second lane is the polyplex (polymer/pDNA ratio 2) treated with DNase I and was found in the gel pocket as reported previously. The third lane is PGA treated sample which is found migrated in the gel as the native pDNA.

In Vitro Cell Viability Studies. Cell viabilities of Man-bPEI-HA conjugates were compared to bPEI-HA and unmodified bPEI using MTT assay. The MTT assay was employed to evaluate relative cell viability over a wide range of concentrations of uncomplexed polymers for incubation times of 4 and 24 h. MTT assay demonstrated that cellular viability was closely dependent on the HA conjugation (Figure 7A,B). Mannosylation of bPEI-HA did not affect the cell viability of RAW 264.7 cells, and nearly comparable cell viabilities were observed in all tested concentrations when compared with bPEI-HA. Unmodified bPEI caused a greater decrease in cell viability which dropped to nearly 5% or less at a concentration of 50 μg/mL. As expected, the cell viability of all conjugates and bPEI was reduced when the incubation time was increased from 4 to 24 h (Figure 7A,B). Similar trends in cell viability were obtained using MTT assay when THP1 cells were treated with Man-bPEI-HA, bPEI-HA, and unmodified bPEI (Supporting Information, Figure 3).
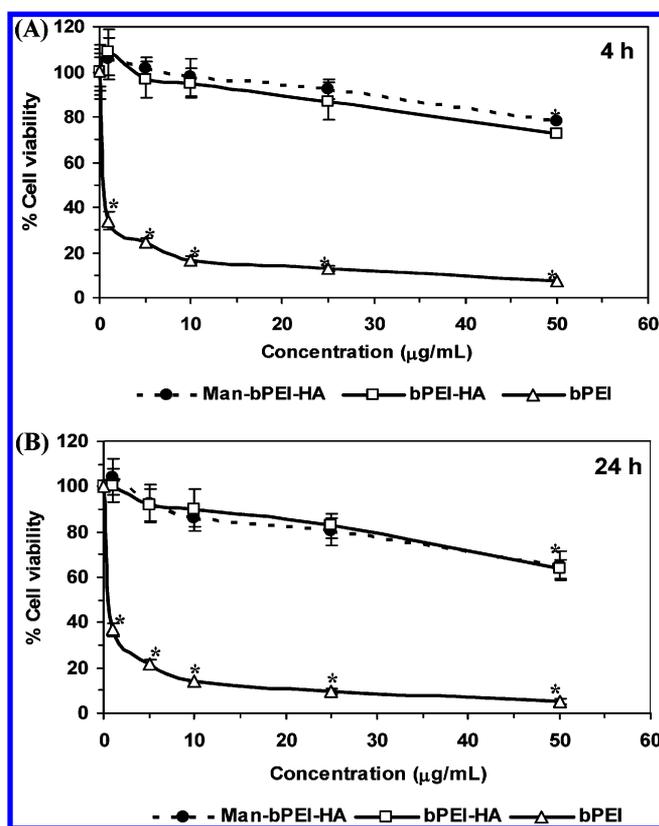
Figure 7. In vitro cell viability of RAW 264.7 cells using a MTT assay by incubating Man-bPEI-HA, bPEI-HA, and unmodified bPEI at different concentrations from 1 to 50 μg/mL for (A) 4 h and (B) 24 h. Data are shown as mean $\pm$ SD ($n = 3$) and considered significantly different if *$P < 0.05$ and **$P < 0.01$.

AlamarBlue assay was performed to evaluate the cellular metabolic activity caused by polyplexes under transfection conditions (4 h) and compared with uncomplexed polymers. Results from the alamarBlue assay revealed that Man-bPEI-HA/pDNA complexes showed greater cellular metabolic activity than polymers alone for 4 h of incubation with RAW 264.7 cells at the same concentration (Supporting Information, Figure 4). For polyplexes, alamarBlue reduction was between 23.75 $\pm$ 1.99% and 17.37 $\pm$ 2.61% and was comparable to control cells that were incubated in complete media (25.21 $\pm$ 1.99%).

Further, comprehensive evaluation of metabolic activity was performed using MTT assay in an in vitro transfection experimental setup. Man-bPEI-HA/pDNA, bPEI-HA/pDNA, and bPEI/pDNA of a different ratio (0.1, 1, 2, 5 and 10) were incubated with cells pretreated with Man-BSA and BSA alone. The Man-bPEI-HA/pDNA complexes have similar cell viability to those of the control (cell alone) except the ratios 5 and 10, both of which showed lower cell viability than that of cell alone and polymer/pDNA ratios 0.1, 1, 2, and 3 (Supporting Information, Figure 5A and B). There was no significant difference in cell viability between Man-bPEI-HA/pDNA and bPEI-HA/pDNA (Supporting Information, Figure 6A and B), whereas the bPEI/pDNA complexes showed lower cell viability than the control, as well as to other ratios of Man-bPEI-HA/pDNA and bPEI-HA/pDNA (Supporting Information, Figure 6A and B). This trend was found to be similar for both RAW 264.7 and THP1 cells. It can also be seen that pretreatment of

cells with Man-BSA and BSA did not cause any adverse effects to cell viability.

Transfection Studies. Transfection studies were performed on RAW 264.7 murine macrophage cells expressing high levels of mannose receptors and on NIH-3T3 mouse fibroblasts lacking this receptor. The transfection efficiency was dependent on cell line and polymer to pDNA weight ratios (Supporting Information, Figure 7A). For RAW 264.7 cells, Man-bPEI-HA showed an increased transfection of luciferase reporter gene compared to unmodified bPEI and bPEI-HA for each weight ratio. At weight ratio 2, Man-bPEI-HA showed significantly higher ($P < 0.01$) reporter gene expression compared to pDNA alone, bPEI-HA, and unmodified bPEI, while differences between the bPEI-HA and bPEI were not significant. Luciferase levels of expression were increased with increasing weight ratio of Man-bPEI-HA and bPEI-HA conjugates to pDNA, and reached the maximum at a weight ratio of 2. Thereafter, increasing the weight ratio clearly decreased the level of expression of luciferase reporter gene, although the difference was not significant.

The target specificity of mannosylated nanohybrids was evaluated in the nonspecific cell types, i.e., NIH3T3 mouse fibroblast cells which lack in mannose receptors. Compared to unmodified bPEI, the transfection efficiency of Man-bPEI-HA polyplexes in NIH-3T3 mouse fibroblasts was decreased for all weight ratios tested. Polyplexes with Man-bPEI-HA, bPEI-HA, and unmodified bPEI were investigated at polymer to DNA weight ratios ranging from 0.5 to 10 (Supporting Information, Figure 7B). At lower weight ratios (≤ 2), luciferase expression mediated by Man-bPEI-HA conjugates was lower with unmodified bPEI in NIH3T3 cells. At higher weight ratios (≥ 2), the luciferase expression was not significantly different for all the conjugates when compared with unmodified bPEI. However, it was observed that the higher the polymer to pDNA weight ratio, the greater the reduction in luciferase reporter gene expression. When the transfection results in RAW 264.7 and NIH-3T3 for Man-bPEI-HA at weight ratio 2 were compared, no significant differences in transfection level were observed.

The competition studies with cells pretreated with Man-BSA showed a significant reduction (~ 2 times) in the GLuc expression level of Man-bPEI-HA/pCMV-GLuc compared to that of cells pretreated with BSA alone in the case of both RAW 264.7 and THP1 cells (Figure 8A,B). Also, the cells pretreated with BSA alone showed an almost a similar level of expression to that of untreated cells, as previously shown in the case of RAW 264.7 cells. On the other hand, bPEI-HA/pCMV-GLuc and bPEI /pCMV-GLuc showed no significant difference in the GLuc expression level in between Man-BSA and BSA in the case of both cell types (Supporting Information, Figure 8A and B).

The competitive assay was further carried out using pCMV-GFP as a reporter gene and taking Man-bPEI-HA/pCMV-GLuc of ratio 2 for this specific study. The FACS analysis showed a reduction (2.5 times) in percentage of cells expressing GFP in the case of cells treated with Man-BSA than that of BSA treated cells. A similar trend was found in both RAW 264.7 and THP1 cells (Figure 9). Imaging of cellular expression of GFP was performed using confocal microscopy (Supporting Information, Figures 9 and 10). The results revealed that the Man-bPEI-HA complex was able to mediate transfection of RAW 264.7 and THP1 cells and more intense green colored cells were observed. The green fluorescence was
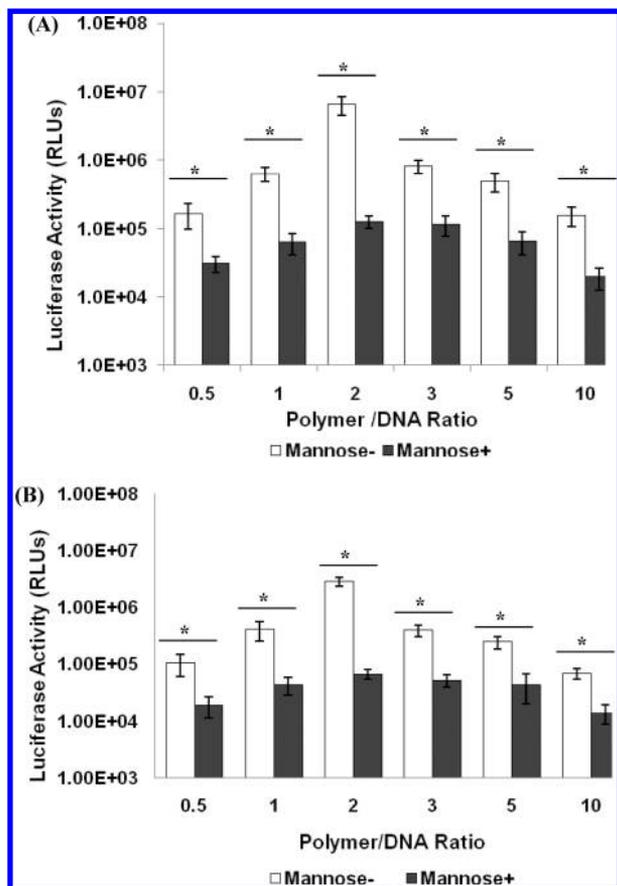
Figure 8. In vitro *gaussia* luciferase gene expression in (A) RAW 264.7 and (B) THP1 cells using reporter plasmid pCMV-GLuc. Cells were pretreated with Man-BSA (a specific competitor) and BSA (a nonspecific competitor) for 30 min followed by incubation with Man-bPEI-HA complexes at different weight ratios, and after 48 h, supernatant was collected and assayed for reporter luciferase activity. Data are shown as mean ± SD ($n = 3$) and considered significant if *$P < 0.05$.

seen to be distributed homogenously throughout the cells (Supporting Information, Figures 9 and 10).

Fluorescence Colocalization Studies. The Man-bPEI-HA/pDNA complexes can be observed inside RAW 264.7 and
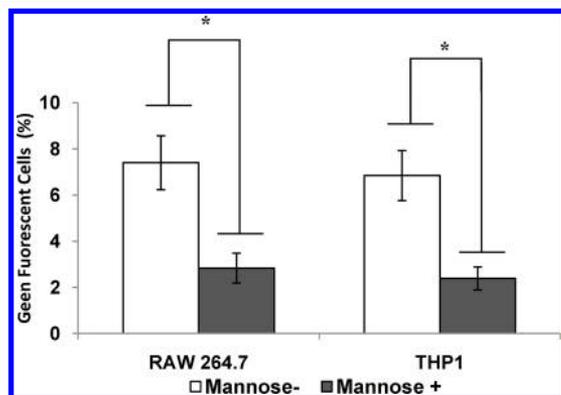
Figure 9. In vitro transfection analysis of in RAW 264.7 and THP1 cells using pCMV-GFP and Man-bPEI-HA at polymer to DNA weight ratio of 2. FACS analysis of GFP expressing RAW 264.7 and THP1 cells preincubated with either Man-BSA or BSA. Data are shown as mean ± SD ($n = 3$) and considered significant if *$P < 0.05$.

THP1 cells (Figure 10A,B). The merged images of polyplex stained with FITC and lysosomes stained with Lysotracker Blue showed colocalization of the polyplexes inside lysosomes in both cells. Man-BSA treated cells showed less fluorescence intensity of FITC labeled polyplexes than that of BSA treated cells. The internalization pattern was found to be similar in both the cell types.

■ DISCUSSION

Macrophages play a crucial role in tissue maintenance, immune regulation, and pathogen control and thus are crucial targets for gene therapy.[37] Several attempts to deliver genes have been made using electroporation, lipoplex, or polymer to modulate the function and dysfunction of macrophages.[38−43] However, gene transfection in macrophages has proven difficult.

In the present study, we developed functionalized hyaluronan based nanohybrids, i.e., Man-bPEI-HA, for enhanced gene delivery in both murine and human macrophages in vitro. As mannose receptors are overexpressed in macrophages, and this expression mediates mannose receptor endocytosis, this phenomenon could be used in designing targeted gene therapy applications. Development of an efficient mannosylated non-viral carrier is potentially a powerful approach for macrophage-specific gene delivery.[44] In a previous study, low molecular weight HA coated PEI/pDNA polyplexes showed reduced cytotoxicity and enhanced CD44 receptor mediated cellular uptake.[14] Therefore, to obtain both gene transfection activity and macrophage cell specificity Man-bPEI-HA nanohybrids were developed using mannopyranosylphenyl isothiocynate and HA conjugate of bPEI. As shown in Figure 1, N—H and thionyl (C═S) stretching peaks appeared in the regions of 3250−3350 and 1550−1620 cm^{-1}, respectively, indicating that bPEI was coupled to HA and mannose residue was grafted to the bPEI-HA conjugates. ^{1}H NMR spectra further proves the conjugation of Man-bPEI-HA with the final ^{1}H NMR spectrum containing three peaks corresponding to HA (1.9 ppm), PEI (2.5−3.2 ppm), and mannose (7 ppm). Previous studies reported that a lower degree of substitution enhanced gene transfection in a hepatocyte cell culture model (HepG2) expressing the asialoglycoprotein receptors.[45] As shown in Figure 3A, ninhydrin assay revealed that the unmodified bPEI possesses a higher number of primary amines than do bPEI-HA and Man-bPEI-HA nanohybrids. The degree of substitution for mannose residue in Man-bPEI-HA was calculated and found to be approximately 8%. The mechanism of polyethyleneimine-based gene transfection is well-studied.[4,6,7] As shown in (Supporting Information, Figure 1), the Man-bPEI-HA nano-hybrids have a buffering ability comparable to bPEI, which might be essential for endosomal escape of pDNA to enter the nucleus for good transfection ability. The binding ability of Man-bPEI-HA with pDNA was also confirmed using gel retardation and UV spectrophotometry studies (Figure 4 and Supporting Information, Figure 2).

Surface charge is also a key property for a nonviral gene delivery system, as it can influence stability, cell adhesion, and transfection efficiency.[46,47] The presence of a net positive surface charge on the complex could lead to attachment to cell surfaces that have a negative charge at neutral pH.[48] Man-bPEI-HA/pDNA complexes were also characterized for size and surface charge distribution, and it was found that the polyplexes were nanometric in size having net positive charge on their surfaces (Figure 5 and Supporting Information, Table 1). The zeta potential analysis of mannosylated nanohybrids/pDNA
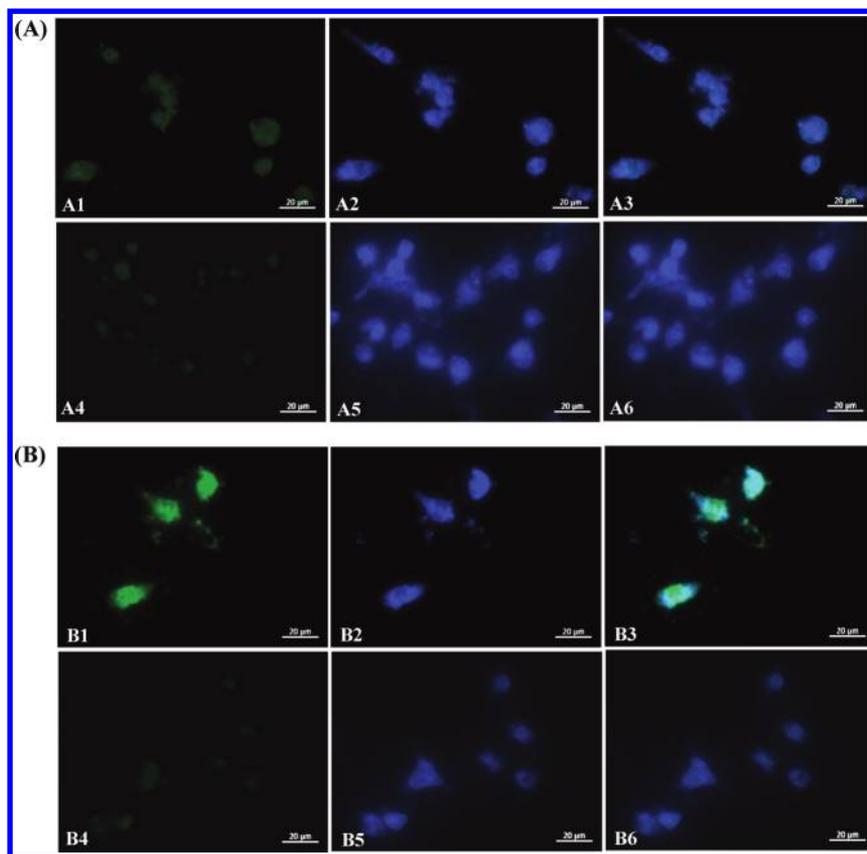
Figure 10. Colocalization study of FITC labeled Man-bPEI-HA/pDNA in (A) THP1 and (B) RAW 264.7 cells. THP1 cells preincubated with (A1–A3) BSA and (A4-A6) Man-BSA. RAW 264.7 cells pretreated with (B1−B3) and (B4−B6) Man-BSA. (A1, A4, B1, B4) FITC labeled polyplex inside the cells tinted as green, (A2, A5, B2, B5) lysosome stained with lysotracker blue, and (A3, A6, B3, B6) composite images of green-tinted polyplexes and blue lysosomes.

complexes at various weight ratios are shown in Figure 5. The Man-bPEI-HA/pDNA complexes of weight ratio 0.5 show a negative zeta potential, whereas complexes with a weight ratio from 1 to 10 show increasing positive charges as the weight ratio is increased. The weight ratio of HA to bPEI used in the present study was 1:5, which is the primary cause of the net positive charge of these conjugates. HA is an anionic polymer that has a carboxylic group. The conjugation of HA to PEI using EDC cross-linking chemistry results in the reduction of the primary amino groups (Figure 3) as COOH of HA couples to primary amino groups of bPEI. Figure 5 represents the size and surface charge of polyplexes (polymer-pDNA complexes) at different weight ratio of polymer to pDNA. As polymer to pDNA weight ratio increases, the net surface charge of polyplexes increases. These results indicate that mannosylated nanohybrids are able to facilitate gene transfer due to their advantageous particle sizes and surface charges. In previous studies, protection of plasmid DNA against endonuclease enzyme is a critical parameter for efficient gene transfection.[34] Protection studies revealed that the Man-bPEI-HA/pDNA complexes were stable against DNase I attack once they form complexes at each ratio, whereas naked pDNA was degraded by DNase I (Figure 6A). Further investigations were carried out using PGA. PGA is highly negatively charged, and incubation of polyplex with a high concentration of PGA leads to destabilization of the polymer/pDNA complex by breaking the electrostatic interaction between polymer and pDNA. The agarose gel showed a migrated band similar to the native one when treated with PGA (Figure 6B). These results further

confirmed the previous assumption that pDNA retention in the gel pockets is caused by complexation of Man-bPEI-HA with pDNA, which protected the pDNA from the enzymatic degradation. These results overall indicate that mannosylated nanohybrids/pDNA complexes are sufficiently able to deliver DNA inside the target cells.

In vitro cell viability studies with RAW 264.7 cells revealed that Man-bPEI-HA showed comparable cell viability with bPEI-HA and significantly improved when HA was conjugated with bPEI at both time points, i.e., 4 and 24 h (Figure 7 and Supporting Information, Figure 3). The in vitro cytotoxicity results were consistent with the previous findings that demonstrated the protective effect of HA for bPEI/pDNA complexes.[14] The cell viability of the polyplexes (Man-bPEI-HA, bPEI-HA, and bPEI) when carried out under in vitro transfection conditions showed better cell viability than that of polymer alone (Supporting Information, Figures 5 and 6). The Man-bPEI-HA and bPEI-HA polyplexes showed comparable viability, more than that of bPEI-based polyplexes. It can also been seen that pretreatment of Man-BSA and BSA did not cause any adverse effect on cell viability and thus did not influence the interpretation of cell viability data of the polyplexes. The cell-specificity for mannosylated nanohybrids was also studied using specific cells (RAW 264.7) and nonspecific cells (NIH 3T3) for mannose receptor. The gene transfection studies showed that mannosylated nanohybrids enhanced the gene transfection of reporter gene in RAW 264.7 cells significantly more than the nonmannosylated nanohybrids (Supporting Information, Figure 7). Also, the competitive

transfection study performed on RAW 264.7 and THP1 cells using Man-BSA and BSA showed lower levels of GLuc in the case Man-BSA pretreated cell than those of BSA pretreated cells and proves that the higher transfection efficiency of Man-bPEI-HA is attributable to mannose specific targeting of the nanohybrids (Figure 8). This was further proven in the FACS analysis of *p*CMV-GFP reporter expression analysis, where a smaller percentage of green fluorescent cells was found in the case of Man-BSA pretreated cells than that of BSA treated cells (Figure 9).

To further prove the concept of mannose targeting and understand the mechanism, an internalization study was performed with Man-bPEI-HA/*p*CMV-GLuc complex tethered with FITC and lysosome with lysotracker blue. Both RAW 264.7 and THP1 cells showed higher levels of colocalization of the complexes inside the lysosome (Figure 10A,B). The higher level of colocalization of polyplexes in the case of BSA pretreated cells can be attributed to mannose-specific targeting, whereas the lower level of colocalization of the polyplexes in the case Man-BSA-treated cells can be ascribed to nonspecific phagocytic internalization by the macrophages. Lysosome was chosen for this study because both the mannose receptor mediated endocytosis and nonspecific phagocytosis involve lysosome. The nanohybrid/*p*DNA complex protects the *p*DNA inside the endolysosomal vesicles during the endocytosis process by rupturing the membrane. Our understanding regarding this escape of endolysosomal escape by polyplex is that a relatively cationic polymer becomes protonated inside the endolysosome due to the low pH. The charged form of this molecule cannot cross the membrane. This accumulation of charge causes an osmotic imbalance between different sides of the membrane, thereby inducing swelling of the endolysosome and, consequently, destabilization of the membrane.[49]

CONCLUSIONS

Hyaluronan conjugation with bPEI and subsequent mannosylation influenced the physicochemical properties of nanohybrids as well as that of polyplexes formed with *p*DNA. Man-bPEI-HA conjugates showed DNA binding capability, and thus polyplexes formed are nanometric in size range, having net positive charge. In addition, Man-bPEI-HA/*p*DNA complexes were seen to be stable against DNase I and intracellular degradation. Furthermore, in vitro cytotoxicity of Man-bPEI-HA conjugates was reduced in comparison to bPEI. As a consequence, transfection efficiency was increased in mannose receptor positive murine macrophages and decreased in mannose receptor negative fibroblasts, and a similar pattern was found when the transfection efficiency was characterized using BSA and Man-BSA, where mannose acts as a competitive inhibitor for the mannose receptor and thus gives a reduced transfection efficiency for the Man-BSA than that of BSA pretreated RAW 264.7 and THP1 cells. This enhanced transfection is most likely because of the improved uptake of polyplexes facilitated by a receptor-mediated phenomenon functionalized with mannose, as shown by the competitive internalization studies. Further in vivo studies, cellular interaction, and other blood compatibility studies are needed to show that these mannosylated hyaluronan-based nanohybrids offer potential as macrophage-targeted gene delivery vectors.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental methods involving cell viability and confocal microscopy and results related to physicochemical characterization of the nanohybrid, cell viability, and transfection assays. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: abhay.pandit@nuigalway.ie. Phone: + 353-(0)91-492758. Fax: + 353-(0)91-485585.

Author Contributions

[#]Authors contributed equally

Notes

The authors declare no competing financial interest.

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