Mannosylated Polyethyleneimine-Hyaluronan Nanohybrids for Targeted Gene Delivery to Macrophages

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Methods

AlamarBlue® assay

AlamarBlue® assay was used to assess the cellular metabolic activity of polymers and polymer/DNA complexes. RAW 264.7 cells were seeded at a cell density of $2x10^4$ cells/well in a 96 well tissue culture plate and grown overnight. Uncomplexed Man-bPEI-HA and Man-bPEI-HA/DNA complexes at different concentrations ranging from 1 µg/mL to 50 µg/mL and 1:1 to 50:1 weight ratios respectively were incubated for 4 h. The cells were then washed with Hank's Blank Salt Solution (HBSS) and replaced with 200 µL of fresh HBSS with alamarBlue® (10% v/v). After 3 h incubation at 37 ^oC in 5% CO_2, 200 µL of assay medium was transferred to a 96 well plate, absorbance was read at 550 and 595 nm on a microplate reader (VarioskanFlash-4.00.53) and the percentage reduction of the dye was calculated.

Confocal microscopy studies for reporter gene GFP expression

RAW 264.7 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 (DMEM + 10% FBS + 1% P/S) and 10,000 cells/chamber, incubated at 37 ºC, 5% CO_2. After 24 h of incubation, the medium was replaced with fresh medium and incubated with Man-bPEI-HA/DNA and bPEI-HA/DNA complexes at optimum polymer to DNA 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 ºC, 5% CO_2. Cells were then washed with cold PBS solution and fixed with formaldehyde solution (4% v/v and 2% sucrose in DI water) overnight at 4 ºC. Cells were incubated with rhodamine (Invitrogen, USA) for cell membrane staining, mounted with Vectashield® and imaged with Zeiss LSM 510 Axiovert inverted confocal microscope equipped with Millennia V Tsunami Multiphoton Laser.

Buffering Capacity of Man-bPEI-HA Nanohybrids

Unmodified bPEI, bPEI-HA and Man-bPEI-HA were diluted to a final concentration of 1 mg/ml with 0.1 N NaCl. The solution was adjusted to pH 10.0 before titration proceeded. An aliquot of 20 µl of 0.05 N HCl was successively added into 5 ml of 1 mg/ml polymer solution, and the changes in pH were recorded by a pH meter (Mettler Toledo, UK).

DNA Binding Studies of Man-bPEI-HA Nanohybrids

Man-bPEI-HA/*p*DNA complexes 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. The mixtures were allowed to incubate at room temperature for 30 min. Man-bPEI-HA/DNA complexes were analysed by a UV spectrophotometer (NanoDrop® ND1000 Spectrophotometer, Thermo Scientific USA).

Results

Buffering Capacity of Man-bPEI-HA Nanohybrids

The effectiveness of bPEI based polyplexes has been correlated to the high buffering capacity of bPEI, especially at the slightly acid pH that is found in the endosomal vesicles.[1-3]The buffering capacity of bPEI was compared with the mannosylated and non-mannosylated bPEI-HA and is shown in Fig. 1. Branched PEI displayed a high buffering capacity over a wide range of pH which is consistent with previous findings.[3] In contrast, Man-bPEI-HA and bPEI-HA conjugates displayed almost comparable buffering capacity and lower than the unmodified bPEI. HA alone and distilled water did not display any buffering capacity.

***p*DNA Complexation Studies with Man-bPEI-HA**

UV spectrophotometric analysis was used as one of the methods to confirm the binding of plasmid DNA to mannosylated bPEI-HA at different polymer to *p*DNA ratios (Fig. 2). The

plasmid DNA (control) showed characteristic absorption maxima at 260 nm without any complexing agent. At a lower Man-bPEI-HA/*p*DNA ratio (0.5:1), there was a reduction of absorption peak because of the reduction in the amount of free *p*DNA, and an almost flat absorption peak was observed at 260 nm when the Man-bPEI-HA to *p*DNA weight ratio reached up to one. Moreover, almost the same flat curve was observed when the Man-bPEI-HA to *p*DNA ratio increased to one, possibly because of the absence of free *p*DNA, and this indicated that the entire DNA amount was complexed with Man-bPEI-HA. These results suggested that a Man-bPEI-HA to DNA weight ratio of one was sufficient to complex the plasmid DNA with mannosylated polymers.

In vitro transfection analysis using GFP as a reporter gene

Green fluorescent protein (GFP) expression was also evaluated in RAW 264.7 cells using a reporter gene encoding GFP. Macrophage cells were transfected with Man-bPEI-HA, bPEI-HA conjugates and unmodified bPEI polyplexes at optimum weight ratio of 2. Confocal microscopy revealed that the Man-bPEI-HA complex was able to mediate transfection of RAW 264.7 cells and more intense green colored cells were observed. The green fluorescence was seen to be distributed homogenously throughout the cells. The cells treated with bPEI-HA and unmodified bPEI also showed transfection but at a much lower intensity than that seen with Man-bPEI-HA. GFP+ cells were only seen in clusters and were located near the edge of the wells along with many apparently apoptotic cells (Fig. 8).

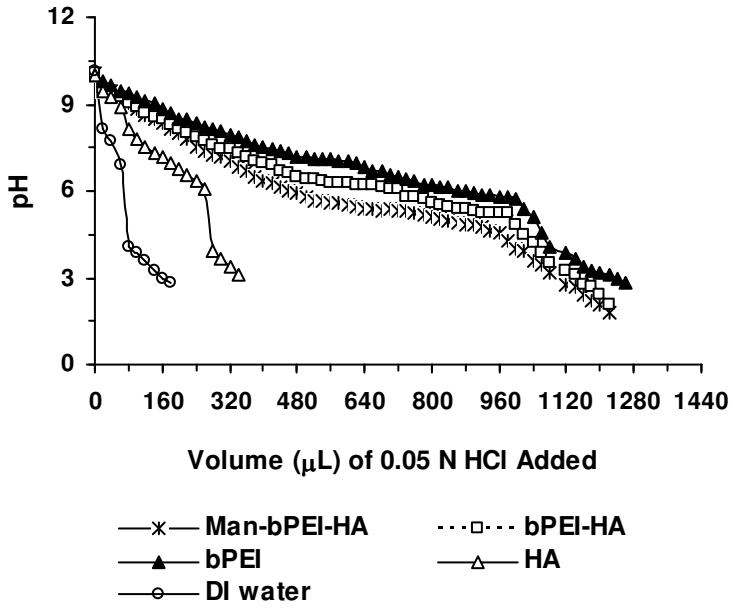


Figure 1. Physicochemical characteristics of Man-bPEI-HA conjugates. Buffering capacity of Man-bPEI-HA, bPEI-HA, unmodified bPEI, HA and deionised water. Samples were accurately weight, dissolved and titrated against each addition of 20 μL of 0.05 N HCl.

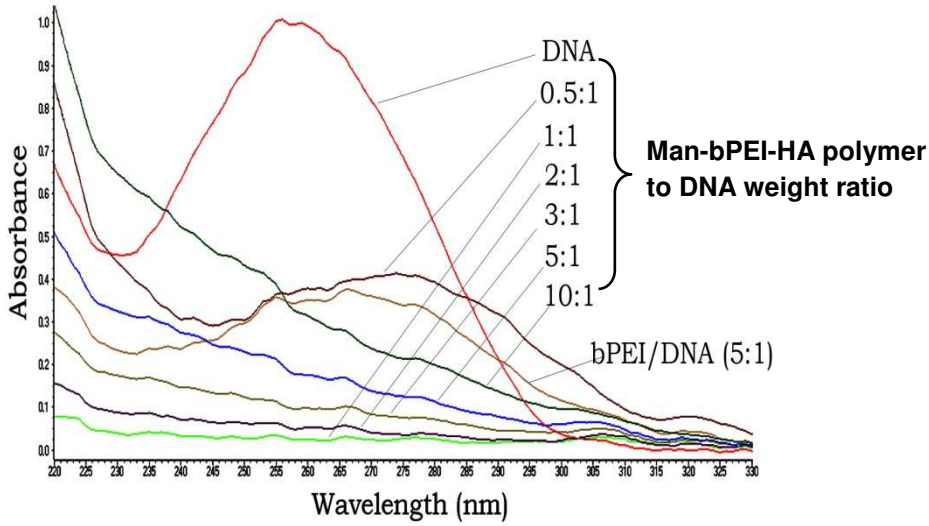


Figure 2. UV spectrophotometric analysis of polymer/*p*DNA complexation studies at different weight ratio of polymer to *p*DNA.

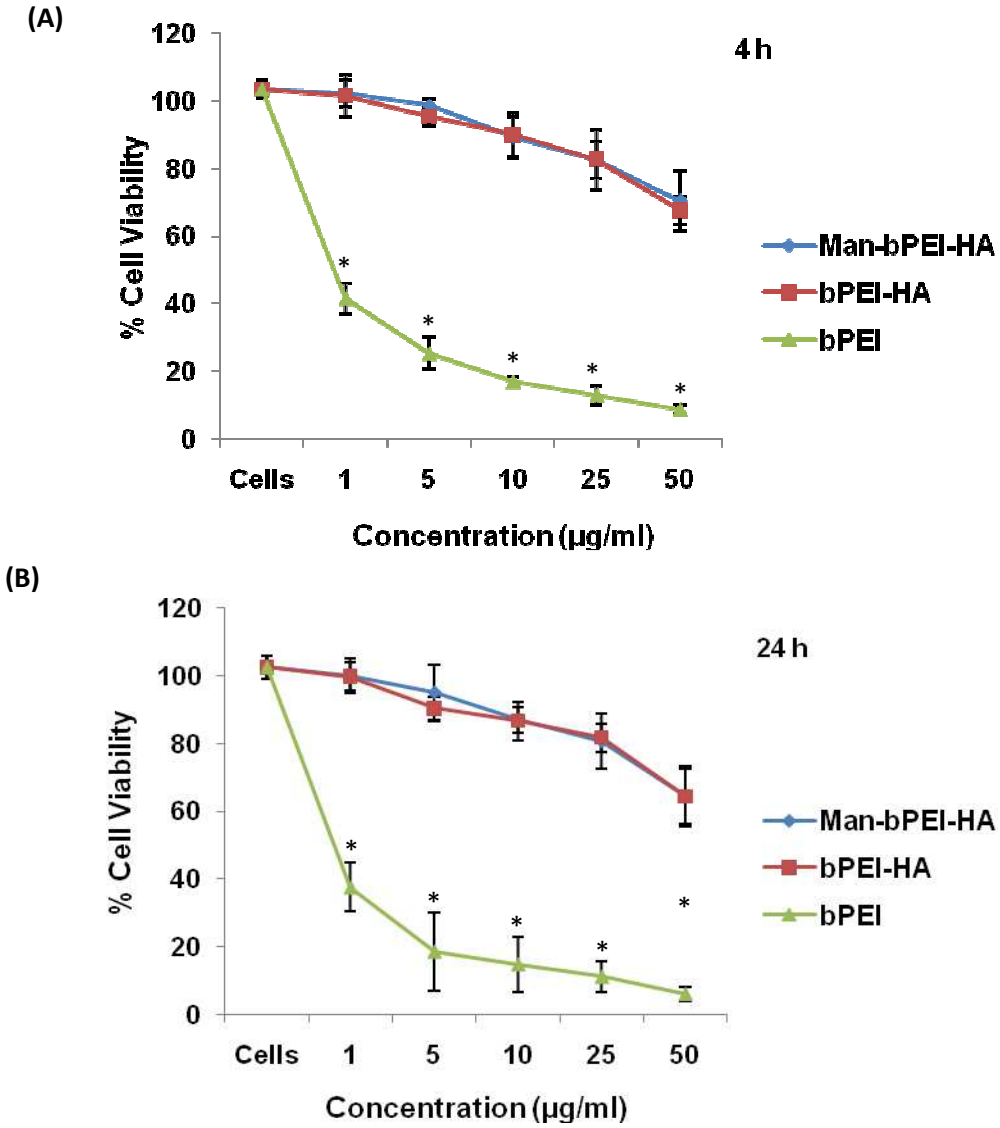


Figure 3. *In vitro* cell viability of THP1 cells using a MTT assay by incubating Man-bPEI-HA, bPEI-HA and unmodified bPEI at different concentrations from 1-50 µg/mL for (A) 4 h and (B) 24 h. Data are shown as Mean ± SD (n =3) and considered significantly different if *P<0.05.

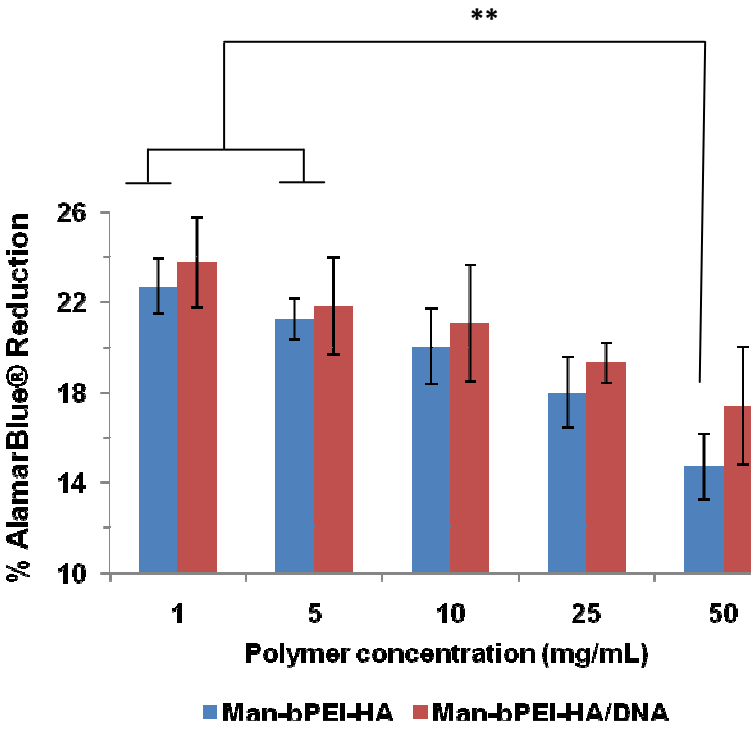


Figure 4. Percent almarBlue® reduction in RAW 264.7 cells when treated with polymers and polymer/DNA complexes for 4 h at same concentration. Data are shown as Mean ± SD (n =3) and considered significantly different if *P<0.05 and **P<0.01.

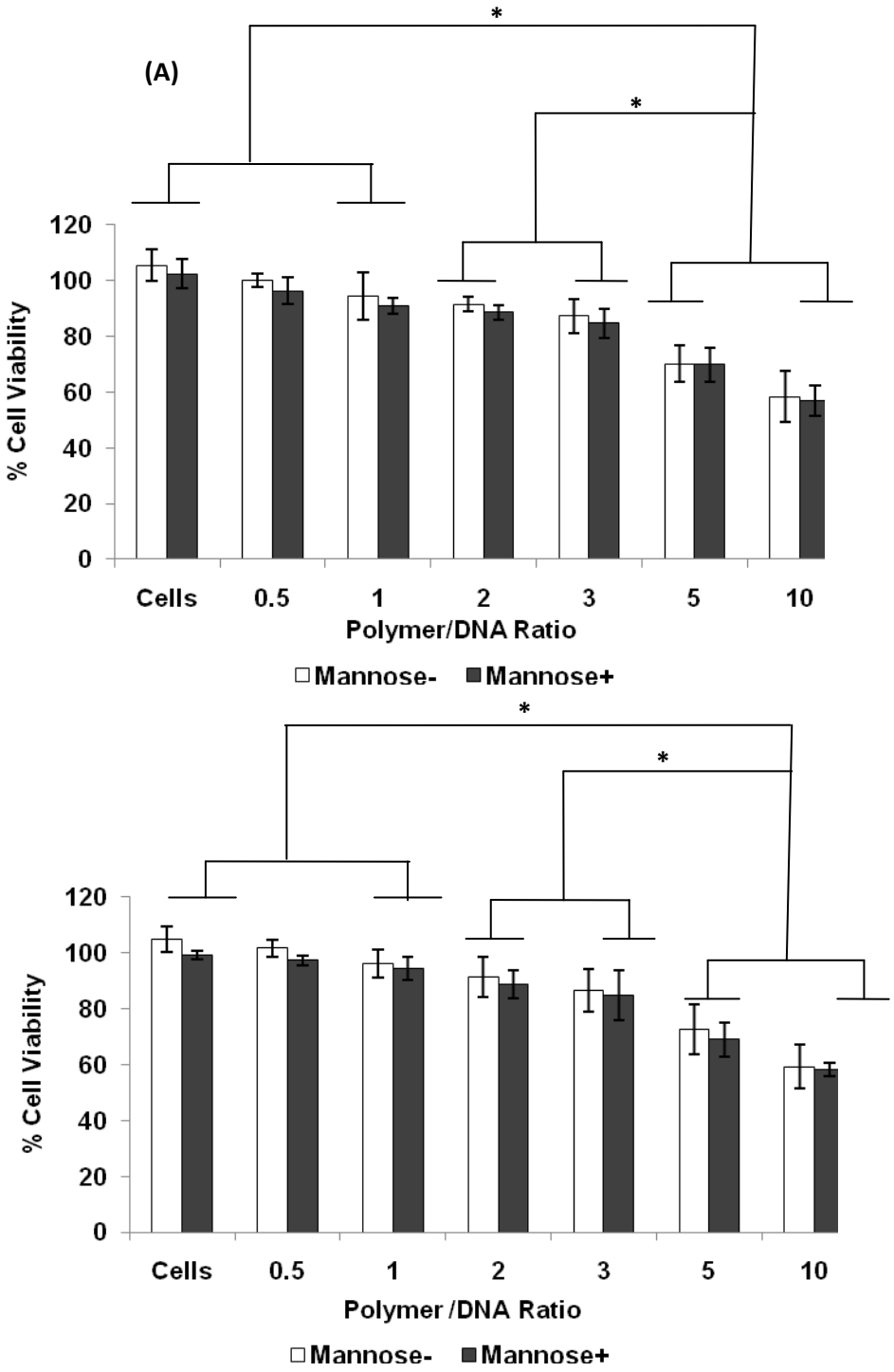


Figure 5. *In vitro* cell viability of (A) RAW 264.7 and (B) THP1 cells. The percent cell viability was determined using a MTT assay and by incubating Man-bPEI-HA/pDNA of different ratios (0.1, 1, 2, 3, 5 and 10) in a transfection conditions. Data are shown as Mean ± SD (n =3) and considered significantly different if $*P<0.05$.

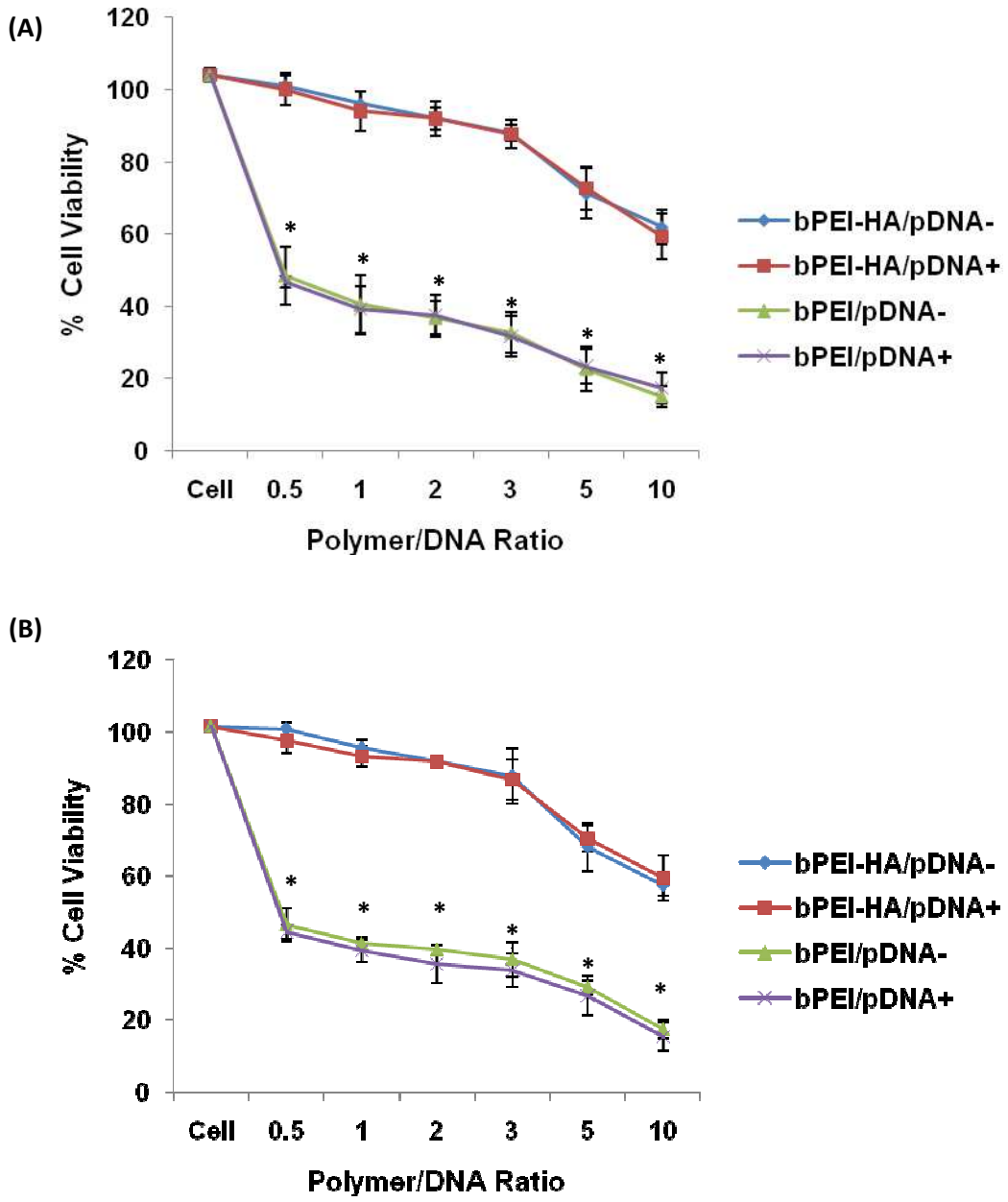


Figure 6. *In vitro* cell viability of (A) RAW 264.7 and (B) THP1 cells. The percent cell viability was determined using a MTT assay and by incubating Man-bPEI /pDNA and bPEI/pDNA of different ratios (0.1, 1, 2, 3, 5 and 10) in a transfection conditions. (-) and (+) signs denote BSA and Man-BSA pre-treatment. Data are shown as Mean ± SD (n =3) and considered significantly different if *P<0.05.

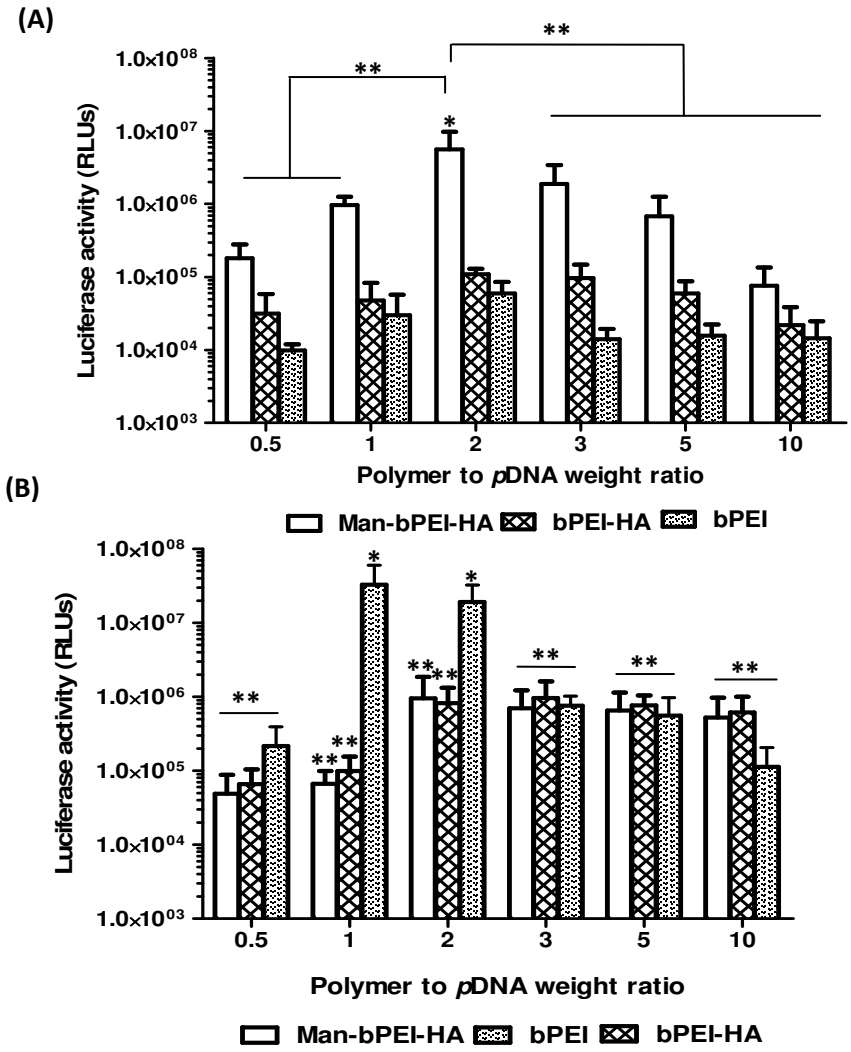
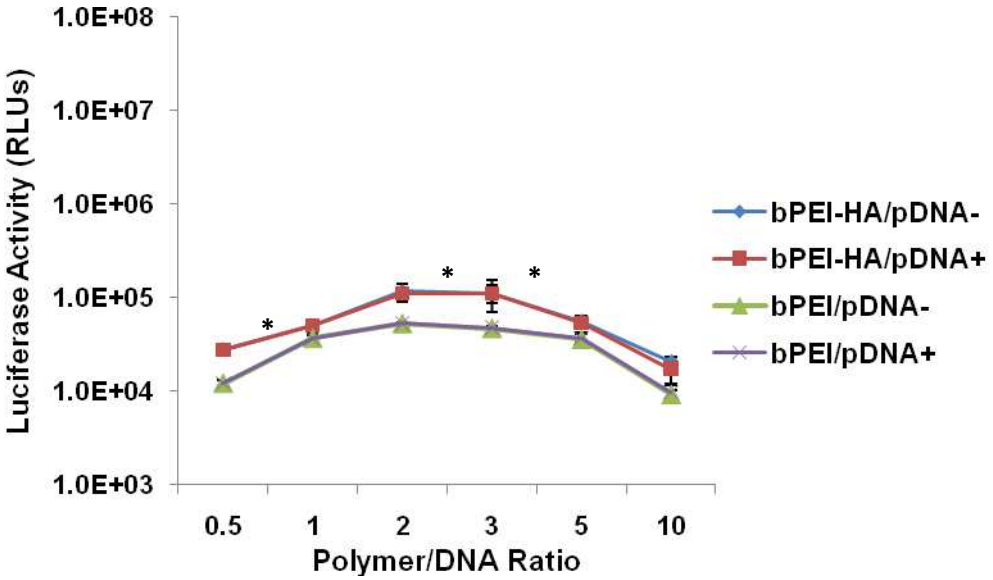


Figure 7. *In vitro gaussia* luciferase gene expression in (A) murine macrophages RAW 264.7 (specific) and (B) NIH3T3 (non-specific) cells using reporter plasmid pCMV-GLuc. Cells were incubated with Man-bPEI-HA, bPEI-HA and unmodified bPEI/*p*DNA complexes at different weight ratios and after 48 h, supernatant was collected and assayed for reporter luciferase activity. Data are shown as Mean ± SD (n=3) and considered significant if *P<0.05and **P<0.01.

(A)



(B)

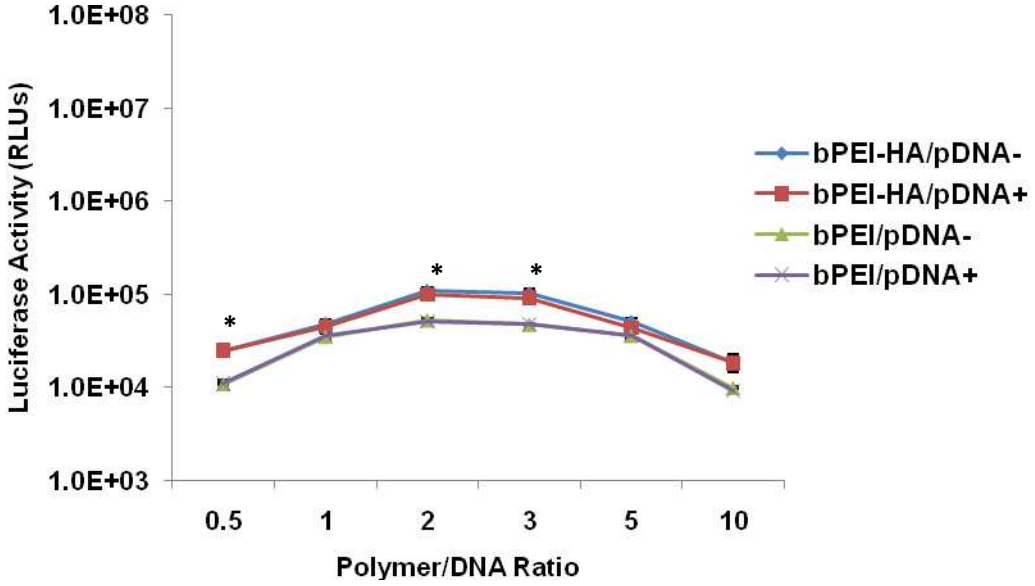


Figure 8. *In vitro gaussia* luciferase gene expression in (A) RAW 264.7 and (B) THP1 cells using reporter plasmid pCMV-GLuc. Cells were pre-treated with Man-BSA (a specific competitor) and BSA (a non-specific competitor) for 30 min followed by incubation with Man-bPEI and bPEI complexes at different 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 $*P<0.05$.

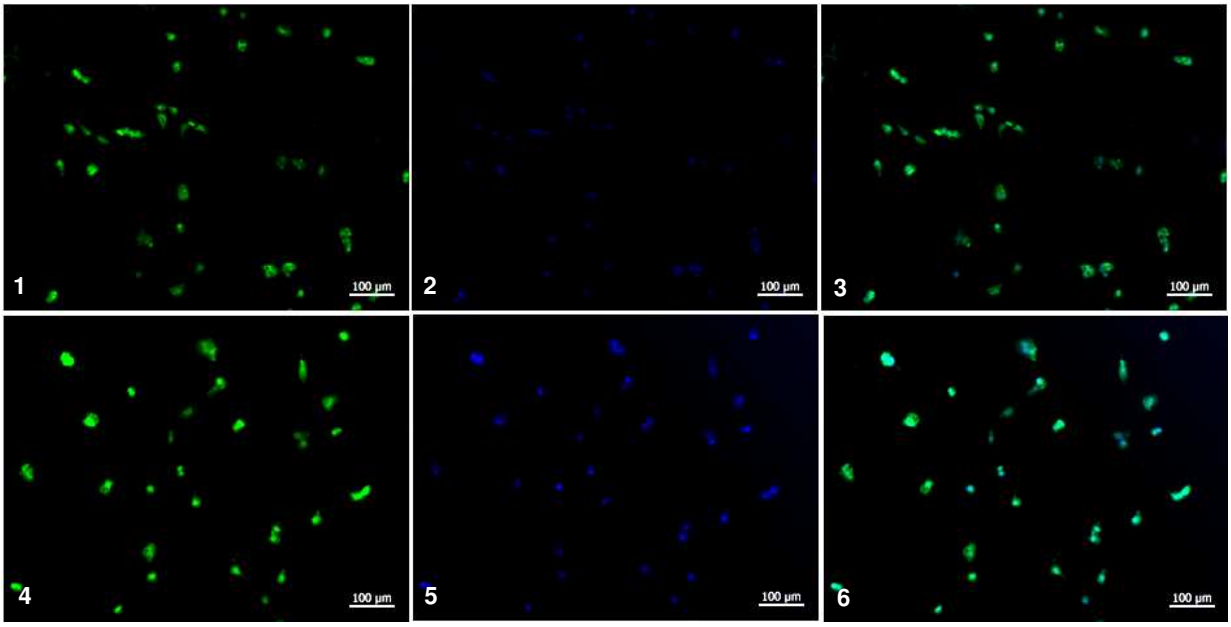


Figure 9. Fluorescence images for GFP transfection (pCMV-GFP) in (1-3) RAW 264.7 and (4-6) THP1 cells. (1 & 4) GFP expressing cells tinted as green, (2 & 5) DAPI nuclear staining of cells as blue and (3 & 6) composite image of GFP expressing green cells and their blue stained nuclei.

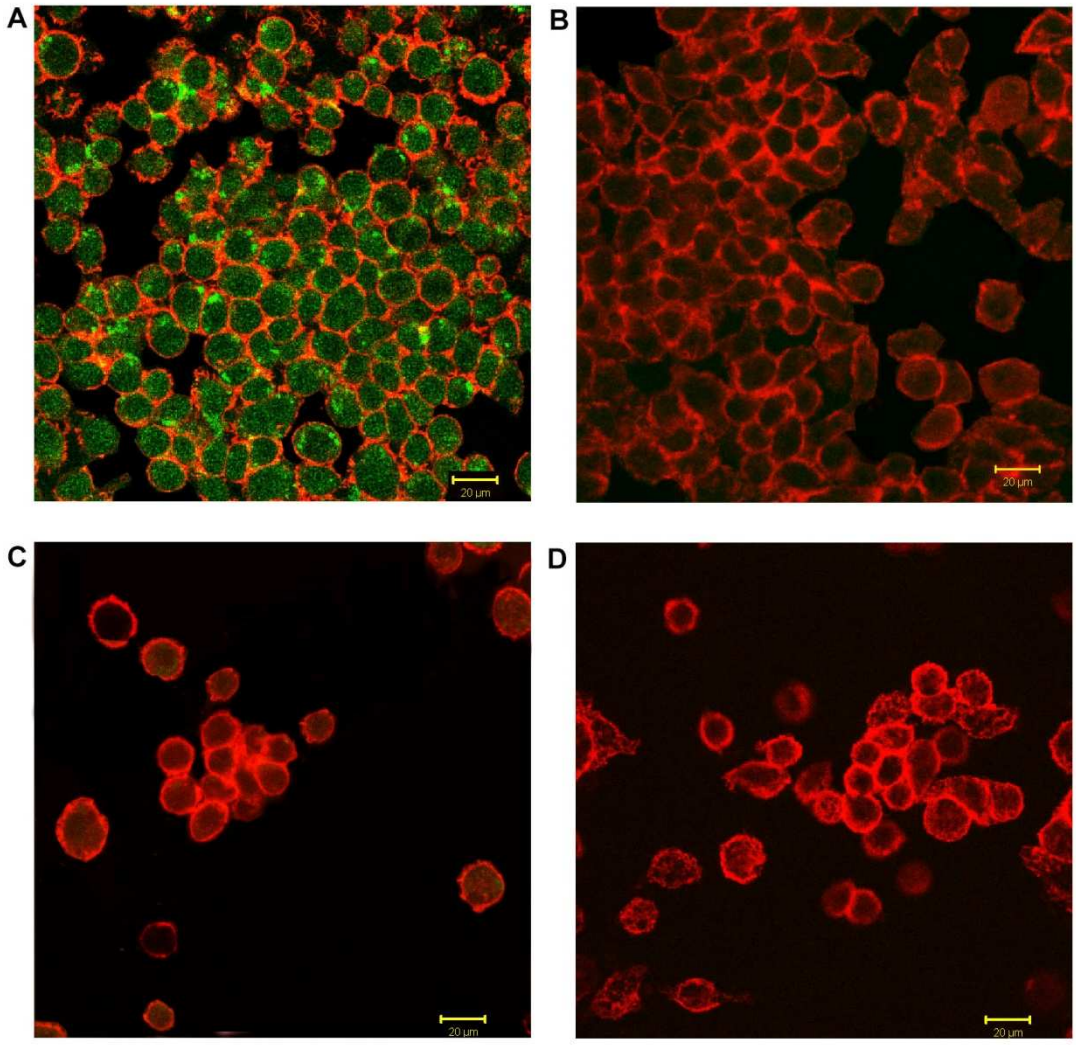


Figure 10. Confocal laser microscopic images for GFP transfection (pCMV-GFP) in macrophages RAW 264.7 cells using (A) Man-bPEI-HA (B) bPEI-HA (C) bPEI at polymer to DNA weight ratio of 2 and (D) DNA alone. Cells were incubated with polyplexes and fixed with paraformaldehyde (4% v/v) and subsequently stained with rhodamine-phalloidin for cytoskeleton staining.

Sample	**Size (nm)**	**Zeta Potential (mV)**
Man-bPEI-HA	1038.7 ± 98.3	34.23 ± 1.3

Table 1. Size and zeta potential values of Man-bPEI-HA alone.

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(3) Tang, M. X., and Szoka, F. C. (1997) The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther. 4*, 823-832.