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European Journal of Pharmaceutics and Biopharmaceutics 65 (2007) 388–397
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

A comparison of the effectiveness of cationic polymers poly-L-lysine (PLL) and polyethylenimine (PEI) for non-viral delivery of plasmid DNA to bone marrow stromal cells (BMSC)

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Received 8 July 2006; accepted in revised form 26 November 2006
Available online 13 December 2006

Abstract

Bone marrow stromal cells (BMSC) represent an important cell phenotype for pursuit of successful gene therapy. Non-viral methods to enable expression of exogenous genes in BMSC will accelerate clinical application of gene therapy, without the concerns associated with the viral means of gene transfer. Towards this end, this study investigated the potential of cationic polymers poly-L-lysine (PLL) and branched polyethylenimine (PEI) as gene carriers for modification of BMSC. Both polymers rapidly (~30 min) condensed a 4.2 kb Enhanced Green Fluorescent Protein (pEGFP-N2) plasmid into 100–200 nm particles. PLL and PEI were both readily internalized with BMSC with >80% of BMSC exhibiting polymer uptake by flow cytometric analysis. The relative uptake of PEI, however, was significantly higher as compared to the PLL. The majority of the BMSC (>60%) exhibited nuclear presence of the polymers as analyzed by fluorescent microscopy. Although both polymers were able to deliver the pEGFP-N2 into the cells under microscopic evaluation, only a small fraction of the cells (<10%) displayed nuclear localization of the plasmid. Consistent with better uptake, PEI gave a higher delivery of pEGFP-N2 into the BMSC, which resulted in a more sustained expression of the model gene EGFP in short-term (7-day) culture. We conclude that both PLL and PEI readily displayed cellular uptake, but PEI was more effective in delivering plasmid DNA intracellularly, which was likely the underlying basis for a more sustained gene expression.

Keywords: Non-viral gene delivery; Cationic polymers; Bone marrow stromal cells; DNA binding; DNA uptake; Flow cytometry

1. Introduction

Cells derived from the bone marrow are being actively pursued in human gene therapy protocols [1,2]. Bone marrow stromal cells (BMSC) are readily harvested from patients, obviating the ethical concerns associated with the use of embryonic stem cells in clinics. The harvested and modified BMSC can be grafted into the host without systemic immunosuppressants, an important consideration that limits application of the therapy to specific patient populations. Significant efforts were recently devoted to BMSC expansion *ex vivo* [3], and it is now a practical procedure to achieve a clinically sufficient cell mass using the current cell expansion technologies. Moreover, BMSC can be manipulated during *ex vivo* culture to direct them into multiple lineages, including osteoblastic, chondrogenic, adipogenic, and myogenic phenotypes [4]. Such a manipulation of BMSC enables construction of functional tissues suitable for tissue replacement in a host. Un-differentiated BMSC, due to their plasticity, may also augment

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doi:10.1016/j.ejpb.2006.11.026

the functioning of intact but diseased tissues, such as the case of BMSC injections to alleviate cardiac failure [5].

The modification of BMSC has been primarily achieved with viral vectors. Some studies reported almost complete modification of BMSC with reporter genes, such as Green Fluorescent Protein (GFP) genes, by using retroviral vectors on mouse-derived (96–98%, [6]), and rat-derived BMSC (90–95%, [7]). Other studies reported lower extent of GFP transfections (based on flow cytometric analysis) by retroviral vectors, ranging from relatively low levels of ~5% for mouse BMSC [8], to intermediate levels of 20–30% for mouse BMSC [9,10], to high levels of 60–80% for mouse, canine and human BMSC [10–12]. Other viral vectors, such as lentiviral and adenoviral vectors, similarly exhibited a wide range of (25–80%) transfection efficiencies in BMSC [13,14]. Our experience with an adenoviral vector delivery was lower than these reported values (10–15%) [15]. Unlike the viral vectors, non-viral vectors offer better safety profiles in a clinical setting, but little quantitative information was reported on the transfection efficiency of non-viral vectors in BMSC. A ~5% efficiency was reported for a lipid-based (FUGENE™) carrier in one report [16] and a higher transfection efficiency (5–17%) was reported for the liposomal Lipofectamine™ in rat BMSC [17]. A cationized gelatin carrier was not effective for modification of rat BMSC, but cationic polymer polyethylenimine (PEI) provided 4–12% cell modification after 48 h of transfection [17]. These results were indicative of lower effectiveness of the non-viral methods of gene transfer; however, due to heavy reliance on transgene expression as an end-point, these studies yielded little information about the limitations of non-viral methods.

In this study, the potential of cationic polymers to deliver exogenous genes to BMSC was further investigated. The use of polymers as gene carriers, as opposed to lipid-based carriers, might offer an important advantage if one wishes to engineer the carrier properties to further control the intracellular trafficking of polymers, for example, to facilitate endosomal escape or to protect against intracellular nucleases. Two cationic polymers commonly used for delivery of plasmid DNA, namely PEI [18] and poly-L-lysine (PLL) [19], were used in this study as the polymeric carriers. Both polymers have a high cationic charge density necessary for DNA condensation, but PEI additional exhibits membrane-perturbing activity necessary for escape of internalized DNA from endosomal compartment. Despite their relatively long history of use for modification of immortal cells, only one study reported the use of PEI on BMSC in a short-term study [17], and no studies reported the use of PLL on BMSC. This study systemically compared the cellular internalization of both polymers, as well as their ability to transport a model plasmid (pEGFP-N2) into the BMSC. The relative effectiveness of PEI and PLL to enable EGFP expression was compared in short-term (~7-day) culture. Our results indicated a more preferential uptake of PEI by BMSC, as well as a better delivery of plasmid DNA intracellularly, resulting in a more sustained expression of the model gene EGFP.

2. Materials and methods

2.1. Materials

Branched PEI (25 kDa), PLL hydrobromide (25 kDa), 5% (w/v) 2,4,6-trinitrobenzosulfonic acid (TNBS), Hanks' balanced salt solution (HBSS), and trypsin/EDTA were obtained from SIGMA (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM; high glucose with L-glutamine), penicillin (10,000 U/mL), streptomycin (10,000 μg/mL) and Lipofectamine-2000™ were from Invitrogen (Carlsbad, CA). Dialysis tubing with a MW cut-off of 12–14 kDa was purchased from Spectrum Laboratories (Gardena, CA). Fetal Bovine Serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). A succinimide ester of Cy5.5 (Cy5.5-NHS) used for labeling of plasmid DNA was obtained from AMERSHAM (St. Laurence, QC). Fluorescein isothiocyanate (FITC) and AlexaFluor-350 (AF-350) were purchased from PIERCE (Rockford, IL) and Molecular Probes (Portland, OR), respectively. A 4.7 kb plasmid incorporating an enhanced green fluorescent protein (pEGFP-N2) and a kanamycin resistance gene was obtained from BD Biosciences, and replicated in kanamycin resistant DH5-α *E. coli* strain grown in Luria–Bertani medium [15]. The purified plasmid was dissolved in ddH_2O at 0.4 mg/mL.

2.2. Atomic force microscopy (AFM)

The MultiMode scanning probe microscope (Digital Instruments Inc., Santa Barbara, CA) was used for all AFM studies. A large-area scanner (J type) with a maximum *xy* scan range of 125×125 μm and a *z* vertical range of 5 μm was used, except for imaging naked plasmid DNA, where a small-area scanner (A type) with a maximum *xy* scan range of 0.4×0.4 μm and a *z* vertical range of 0.4 μm was used for higher resolution. Single crystal silicon cantilevers were cleaned by exposure to high intensity UV light for 3 min before use. The oscillation amplitude of the scanning tip was registered at 0.5 V and the frequency of the oscillation was in the range of 200–400 kHz. All AFM imaging was conventional ambient tapping mode AFM. The scan rate was typically 1.0–2.0 Hz and the data collection was at 512×512 pixels. Images were processed and analyzed using the Nanoscope III software (V5.12).

To visualize naked pEGFP-N2, the plasmid solution at 0.4 mg/mL was diluted to 2.5 μg/mL with 3 mM NaCl (in ddH_2O), vortexed for 10 s, and 10 μL of this solution was transferred onto freshly cleaved mica. After 3 min, the mica disk was dipped into water and excessive water was removed by filter paper. After drying at room temperature for 30 min, the surface was blow-dried with N_2 (if necessary). To visualize pEGFP-N2/polymer complexes, the plasmid DNA and the polymers were dissolved separately in 3 mM NaCl solution. Calculated amount of plasmid DNA and PEI solutions was transferred to a tube main-

taining a desired PEI/DNA mass ratio with the final DNA concentration of 2.5 μg/mL. The samples were then imaged under tapping mode as described above. Particles with smaller than ~20 nm were ignored since these were also visualized with 3 mM NaCl solution alone and were likely to represent NaCl crystals. An equivalent diameter for the larger particles was measured assuming a spherical geometry.

2.3. BMSC isolation and expansion [15]

The BMSC were isolated from both femurs of ~8-week-old female Sprague–Dawley rats and pooled to obtain a single cell suspension. The marrow was removed by cutting the femur at distal and proximal ends with a bone-cutter, and aspirated using a 5-mL syringe with an 18 G needle. The bone marrow was flushed out with ~15 mL of DMEM containing 10% FBS, 50 μg/L ascorbic acid, 100 U/mL penicillin and 100 μg/L of streptomycin (referred as basic medium). The cells were centrifuged (600*g* for 10 min), suspended in fresh basic medium and seeded on a single 75 cm^2 flask. After a medium change on day 3, the cells were trypsinized on day 7, and expanded on 75 cm^2 flasks (1:4 dilution). The BMSC passaged between 2 and 4 generations were used in this study, and the cells were grown in either 6-well plates (Corning), or on 2 cm × 2 cm glass coverslips (FISHER) in 6-well plates for testing as described below. The osteogenic potential of the BMSC from our laboratory was described in separate publications [20,21], and will not be further described here.

2.4. Cell uptake of polymers and pEGFP-N2 by fluorescent microscopy

The PEI and PLL were labeled with 1 mM FITC according to the manufacturer's directions, dialyzed against 0.1 M phosphate buffer, and then against ddH_2O to remove the unreacted FITC. TNBS assay was then used to determine the polymer concentrations in the dialyzed samples, as described [22]. The cells grown on coverslips (in 6-well plates with 2 mL medium) were rinsed with HBSS (2×) and incubated with 3 μg/mL FITC-labeled polymers at 37 or 4 °C. After 3 h, the medium was removed, and the cells were fixed with 70% alcohol. Cells treated with PLL were stained with 0.5% Coomassie blue (in 70% methanol, 10% acetic acid), but not PEI treated cells since this abolished the fluorescence in cells. The presence of FITC-labeled polymers in BMSC was investigated using a epifluorescent microscope (LEICA DM-RL), and cell counts were manually taken (~100 for each treatment group) to determine (i) the percentage of cells displaying FITC-fluorescence and (ii) the percentage of cells displaying FITC-fluorescence at the nucleus.

A similar procedure was used for investigating the cellular uptake of plasmid DNA. The pEGFP-N2 was labeled with 1 mM AF-350 or FITC according to the manufacturer's directions, dialyzed against 0.1 M phosphate buffer, and then against ddH_2O to remove the unreacted AF-350. The labeled pEGFP-N2 (~2 μg) was then incubated with PEI (2 μg) and PLL (12 μg) (P:N ratio of 1:2 and 1:10, respectively) for ~45 min, and the complexes were added to the cells grown on coverslips for 3 h. The chosen plasmid:polymer ratios were based on agarose gel-electrophoresis analysis of the complexes, which corresponded to minimal ratios for complete plasmid complexation. Cells were then visualized with the epifluorescent microscope as above.

2.5. Cell uptake of polymers and pEGFP-N2 by flow cytometry

To quantitatively assess polymer and pEGFP-N2 uptake by BMSC, FITC-labeled PLL and PEI (1–9 μg/mL) were incubated with BMSC in 6-well plates (in 2 mL basic medium/well) for ~24 h. After removing the polymer-containing media, the cells were washed with HBSS, trypsinized, centrifuged, and suspended in HBSS with 3.7% formalin for flow cytometric analysis ($\lambda_{ex} = 488$ nm). The instrument threshold for the negative control sample (i.e., BMSC incubated with no polymers) was setup at ~1% level. The percentage of cells exhibiting FITC-fluorescence beyond this threshold value was calculated as a function of polymer concentration in the medium.

For assessment of pEGFP-N2 uptake, 20 μg of pEGFP-N2 solution (in 300 μL ddH_2O) was incubated with 100 μL of Cy5.5-NHS dye solution (1 mg in 300 μL DMSO), and allowed to react for 2 h at room temperature. The reaction was stopped by extensive dialysis against TBE buffer. 5 μL of this solution was mixed with 1, 3 and 9 μg/mL PEI and PLL in 150 mM NaCl, and incubated for 30 min before addition to cells in 6-well plates (2 mL basic medium/well) for 20 h. After removing the pEGFP-N2-containing media, the cells were washed with HBSS, trypsinized, centrifuged, and suspended in HBSS with 3.7% formalin for flow cytometric analysis ($\lambda_{ex} = 635$ nm). The instrument was calibrated so that the negative control sample (i.e., pEGFP-N2 incubated with BMSC without any polymeric carrier) gave ~1% cell uptake. The percentage of cells exhibiting Cy5.5-fluorescence beyond this threshold value was calculated as a function of polymer concentration in the medium.

2.6. Transfection of BMSC

The polymer/pEGFP-N2 polyplexes used for transfections were prepared by mixing 0.4 mg/mL pEGFP-N2 (in ddH_2O) with 1 mg/mL polymer solutions (in ddH_2O), and bringing the volume to 50 μL with 150 mM NaCl. The polyplexes for each transfection contained 3 μg of pEGFP-N2, and 2, 6 and 18 μg of polymer (either PLL or PEI) in 2 mL medium. After 30 min of complex formation, the polyplexes were added to the cells grown on 6-well plates. The BMSC were incubated for either 8 or 48 h with the transfection reagents, after which the cells were either

trypsinized for assessment of EGFP expression, or the medium was replaced with fresh medium for longer time cultures. Flow cytometry was performed on a BD FACscan where the cell fluorescence was detected by $\lambda_{ex} = 488$ nm for EGFP expression. The instrument settings were calibrated for each run so as to obtain a background level of EGFP expression of ~1% for control samples (i.e., cells incubated with pEGFP-N2 alone without any carrier). An aliquot of the cell suspension used for flow cytometry was manually counted with a hemocytometer to obtain total number of cells recovered from the wells.

2.7. Statistical analysis

Where indicated, all results are summarized as means ± standard deviation, and statistical variations ($p < 0.05$) between the group means were analyzed by the Student's *t*-test.

3. Results and discussion

This study focused on the use of two prototypical cationic polymers, PEI and PLL, for expression of a model gene (EGFP) in primary BMSC. Both polymers have been utilized in the past for gene delivery, but their utility has been usually evaluated in immortal cells that are not clinically useful. BMSC, on the other hand, provides a relevant cell phenotype that can realistically evaluate the potential of non-viral carriers for a clinical application. We are not aware of any studies that compared the two cationic polymers head-to-head for their effectiveness in gene delivery to BMSC. This study, therefore, specifically focused on evaluating the interactions of the polymers with the BMSC, and assessing their ability to deliver extracellular plasmid DNA intracellularly as a means to understand their effectiveness in transgene expression.

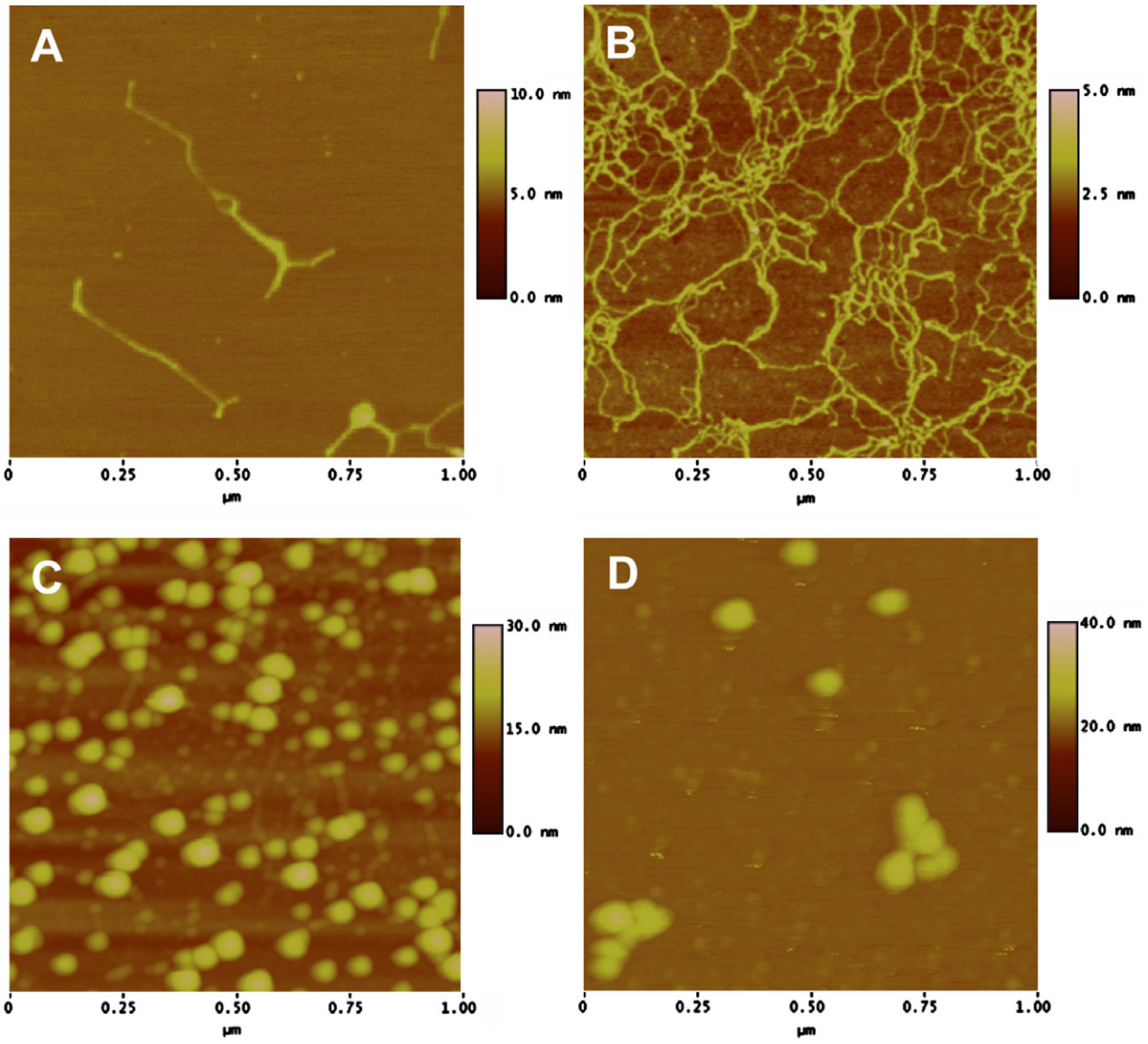
3.1. pEGFP-N2 binding by PEI and PLL

The ability of PEI and PLL to condense the circular pEGFP-N2 plasmid was investigated by AFM. Although condensation of plasmid DNA is paramount, mammalian cells display much flexibility in the size of polymer/DNA complexes for uptake; particles from <50 nm to as large as ~1 μm could be internalized by different mechanisms [23]. The pEGFP-N2 alone gave long, string-like morphologies without any sign of compact, spherical structures (Fig. 1A). Using a PEI and pEGFP-N2 mixture with a mass ratio of 1:5, the complexation between the oppositely charged molecules was investigated after 1, 10 and 30 min of mixing of the macromolecules. After 1 min, a network of string-like structures was evident, whose density was considerably higher than the structures seen with pEGFP-N2 alone (Fig. 1B). Presumably, the presence of the polymer resulted in better retention of the pEGFP-N2 on anionic mice surfaces. At 10 min, a relatively high-density of compact, spherical structures was visible (Fig. 1C) and, after 30 min, a lower density of condensed structures was present (Fig. 1D). There was no evidence of string-like pEGFP-2 molecules as was seen in the absence of cationic polymers. The sizes of the observed spherical particles were dependent on the pEGFP-N2:PEI ratio (see Table in Fig. 1); a general reduction in particle size (from ~240 to ~100 nm) was observed with higher PEI concentrations used to form the complexes. The size range observed under AFM was similar to those of other studies reported by independent investigators; for example, Twaites et al. [24] and Petersen et al. [25] reported plasmid/PEI particles of 60–100 nm (N:P > 2:1) and 80–100 (N:P ~ 9), respectively, under the low ionic salt conditions similar to ours. Prolonged (>30 min) incubation of the plasmid DNA and the polymer was expected to led to a gradually increasing particle size [26], but this issue was not further explored in this study. All particles utilized in this study were formed after 30–45 min of complexation before incubation with the cells.

The particle sizes obtained by complexing pEGFP-N2 with PLL were also similar; an average particle size of ~120 nm with a range of 80–200 nm was obtained after 30 min of complexation (not shown). The distribution of the particles was uniform over this size range, with no apparent preferential size range. A greater degree of heterogeneity was reported by Chan et al. [27] for the PLL/plasmid DNA particles, ranging from ~20 nm to as large as 800 nm (depending on N:P ratio). Miyata et al. [28], on the other hand, reported PLL/DNA particles in the 80–300 nm range. Unlike tursoid-like particles reported in some studies, our particles were all spherical and compact. Taken together, it was evident that the both polymers used in this study were able to condense the chosen plasmid DNA into a size range similar to the previously reported values, and more importantly, sizes which are suitable for cellular uptake.

3.2. Cellular uptake of polymers

To determine the percentage of BMSC displaying polymer uptake, FITC-labeled PEI and PLL were incubated with the cells and the percentage of FITC-fluorescent cells was determined by the flow-cytometry. Almost all of the cells (80–100%) exhibited PLL and PEI uptake after a 24-h incubation period with the polymers (Fig. 2A). This was the case for all polymer concentrations tested (1–10 μg/mL), indicating an effective uptake even at low polymer concentrations in serum-protein rich medium. The extent of polymer uptake by the BMSC, given by the average fluorescence for the FITC-positive cells, was directly proportional to the polymer concentration in the medium (Fig. 2B), as expected. Although PEI-incubated cells had a higher fluorescence in this analysis, it was not appropriate to interpret it as a higher uptake due to differences in FITC-labeling efficiencies of the two polymers. To compare the relative propensity of the two polymers to be internalized, the intracellular fluorescence values (from flow cytom-



pEGFP-N2: PEI ratio	Mean Size	Size Range
1 : 8	240 nm	190 – 270 nm
1 : 4	230 nm	180 – 310 nm
1 : 2	360 nm	190 – 490 nm
1 : 1	170 nm	130 – 240 nm
1 : 0.5	100 nm	60 – 160 nm

Fig. 1. Atomic force microscopic investigation of pEGFP-N2 condensation by PEI. pEGFP-N2 without any polymers (A) and pEGFP-N2 incubated with PEI for 1, 10 and 30 min (B, C, and D, respectively). The mean size and the size range of the particles format by pEGFP-N2/polymer complexes at different mass ratios were shown in the Table after 30 min of complex formation. Note that the size of the particles formed was reduced at higher pEGFP-N2:PEI mass ratios.

etry) were normalized with the original fluorescence incubated with the cells (from fluorometry). The normalized uptake of the polymers (Fig. 2C) indicated a 30–50 fold higher propensity of the PEI to undergo cell uptake.

A critical issue with the uptake of the polymers was the intracellular localization of the polymer, i.e., cytoplasmic vs. nuclear. To explore this issue, BMSC grown on coverslips were incubated with the FITC-labeled polymers at 37 and 4 °C, and the percentage of cells exhibiting nuclear fluorescence was quantified under an epifluorescent microscope. At 37 °C, almost all cells had visually detectable levels of polymers. With PEI and PLL, $75 \pm 8\%$ and $74 \pm 13\%$ of the cells displayed fluorescence in the nucleus, respectively (Fig. 3). These levels of uptake were consistent with the flow cytometric observations from Fig. 2. The nuclear uptake was significantly reduced to $21 \pm 1\%$ ($p < 0.001$) and $18 \pm 10\%$ ($p < 0.01$) for PEI and PLL, respectively, after incubation of the BMSC at 4 °C. Since endocytosis is effectively reduced by the low temperature incubation, the cellular uptake of polymers appeared to

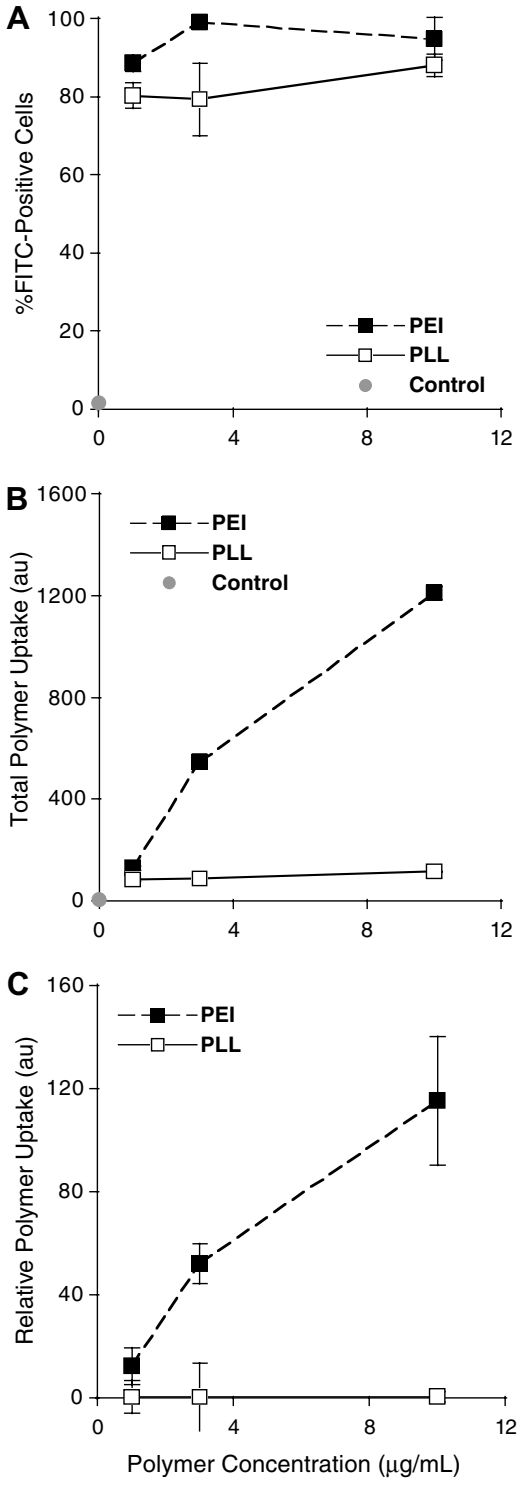


Fig. 2. (A) Uptake of FITC-labeled polymers as determined by flow cytometry. Both PEI and PLL gave >80% cell uptake irrespective of the concentration (1–10 μg/mL) tested for this study. (B) The absolute uptake of polymers (arbitrary units) as a function of polymer concentration. A direct relationship between the absolute uptake and the polymer concentration incubated with the BMSC was evident. (C) Relative polymer uptake as a function of polymer concentration. The relative uptake was calculated by dividing the internalized fluorescence values (from flow cytometry) with the actual amount of fluorescence added to cells (from fluorometry). Note that a relatively higher fraction of the PEI was taken up by the BMSC.

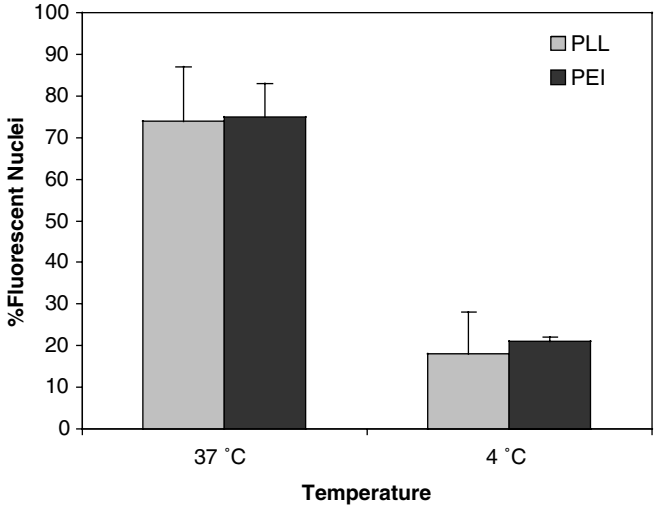


Fig. 3. Quantitation of cells displaying polymer uptake at the nucleus after incubation with polymers at 37 and 4 °C. There were no obvious differences between the two polymers for nuclear localization after polymer uptake at both temperatures.

be strongly dependent on this active internalization process.

These results have indicated a relatively uniform uptake of the polymers by the BMSC. Although BMSC are known to represent a relatively heterogeneous mixture of pluripotent cells, both polymers were internalized and located in the nucleus of the majority of cells. The latter observation was particularly encouraging, since nuclear localization is an essential requirement for long term expression of transgenes [29]. Nuclear localization was expected for the PLL, since peptide-based nuclear localization signals typically incorporate consecutive lysine residues [29]. The PEI, although being a synthetic polycation, was also shown to be capable of crossing the nuclear membrane in immortal cells [30,31]. A 2.5- to 3.5-fold higher nuclear concentration of PEI was estimated as compared to extracellular concentration [30], suggesting active accumulation of this polymer in the nuclear compartment of certain cells. The BMSC, hence, appeared to behave in the similar manner for nuclear binding of PEI as well. Conjugating nuclear targeting peptides to PLL [26] and PEI [32] was shown by others to enhance the expression of delivered genes, presumably due to more effective transport of this carrier to the nuclear compartment. It remains to be seen whether such moieties can further enhance nuclear transport of the polymers in BMSC.

3.3. Cellular uptake of pEGFP-N2 with polymeric carriers

A parallel set of studies was conducted with AF350- and FITC-labeled pEGFP-N2 using epifluorescent microscopy to investigate whether the cationic polymers were able to deliver the exogenous plasmid into the cells. Two different chromophores were utilized to ensure that the labeling technique did not affect assessment of intracellular uptake. Without a polymeric carrier, only a small fraction (<2%) of

the cells contained any internalized pEGFP-N2, irrespective of the chromophore used for labeling (Fig. 4A). This was expected given (i) the non-compact nature of the plasmid DNA in the absence of polymers, and (ii) the incompatibility of the anionic nature of both the cell-surface and the plasmid DNA. With PEI as the carrier, $64 \pm 18\%$ of the cells exhibited plasmid uptake, but only a small percentage of the cells ($7 \pm 12\%$) exhibited nuclear uptake of the AF350-labeled plasmid. A similar result was obtained with the FITC-labeled pEGFP-N2; $79 \pm 20\%$ of the cells exhibited pEGFP-N2 uptake but only $9 \pm 10\%$ of the cells gave nuclear localization of the pEGFP-N2. Since the plasmid uptake was similar with both labeling approaches ($p > 0.05$ for both plasmid uptake and nuclear localization between the two labeling techniques), further experiments to compare the relative effectiveness of PEI and PLL were performed with only AF350-labeled pEGFP-N2 (Fig. 4B). Co-delivery with PLL and PEI resulted in $98 \pm 3\%$ and $67 \pm 7\%$ of BMSC exhibiting pEGFP-N2 uptake, respectively. However, only $8 \pm 6\%$ and $14 \pm 3\%$ cells exhibited nuclear localization of the plasmid with PEI and PLL, respectively ($p > 0.05$). Therefore, this analysis indicated no discernable differences between the two polymers; both polymers delivered the plasmid DNA into majority of cells (>60%), but nuclear localization was displayed in only a small percentage of cells (<10%). The relatively low nuclear delivery was in contrast to the polymer localization, which was readily evident in most cellular nuclei. The plasmid DNA clearly experiences a higher impediment for nuclear location in BMSC. This was, however, not unusual considering the nuclear uptake shown by other cell types, such as immortal ΣCFTE290-cells (~20%, [33]), and primary bronchial epithelial cells (~5%, [33]) were also in the range displayed by the BMSC in this study. It is important to note that this microscopic analysis was based on visual assessment of the fluorescent cell population, and not quantitative levels of fluorescence in the cells. As such, the results provide a measure of the cell population amenable for polymer-mediated plasmid uptake, but do not attest to the quantitative extent of delivery by the polymers.

Complementary information about the effectiveness of the polymers to deliver pEGFP-N2 into BMSC was obtained from flow cytometry. For this, Cy5.5-labeled pEGFP-N2 was formulated with PEI and PLL, and the percentage of cells displaying intracellular uptake of the labeled plasmid was determined. Both polymers exhibited a concentration-dependent increase in the intracellular pEGFP-N2 delivery (Fig. 5). Whereas PLL was effective at polymer concentration of $\geqslant 3$ μg/mL, PEI exhibited an effective delivery of the plasmid even at the lowest concentration tested (1 μg/mL). More importantly, a ~10-fold higher percentage of cells displayed significant pEGFP-N2 uptake when the plasmid was formulated with PEI as compared to PLL. Note that assessment by flow cytometry generally indicated a smaller percentage of cells with the plasmid uptake, except for the plasmid formulated with the highest PEI concentration. This was unlike the good agreement observed when both of these techniques were applied for assessment of polymer uptake. The lower plasmid uptake indicated by flow cytometry was presumably due to lower sensitivity of this assessment technique as compared to direct visual assessment by epifluorescent microscopy. Flow cytometry also relies on the use of trypsinized cells, which might lead to signal reduction from the cells as a result of enzymatic treatment. Nevertheless, a clear difference between the two polymers was evident, with PEI providing a more effective delivery of the extracellular plasmid DNA.

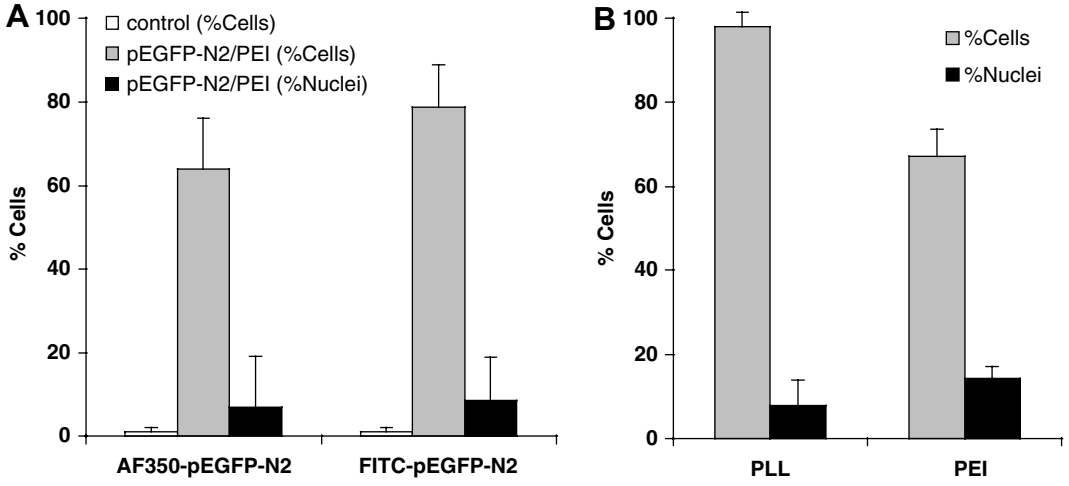


Fig. 4. (A) Microscopic investigation of pEGFP-N2 uptake by BMSC. The cells were incubated with AF-350 or FITC labeled pEGFP-N2 without any polymer (control), or the same labeled pEGFP-N2s complexed with PEI (A). In the absence of PEI, only ~2% of BMSC displayed pEGFP-N2 uptake, whereas >62% of BMSC displayed pEGFP-N2 uptake with the PEI carrier (irrespective of labeling method). However, only a small fraction of BMSC exhibited nuclear uptake of the plasmid. (B) A comparison of pEGFP-N2 delivery by PEI and PLL. Note that both polymers displayed significant pEGFP-N2 uptake with relatively small fraction of BMSC displaying the exogenous plasmid at the nucleus.

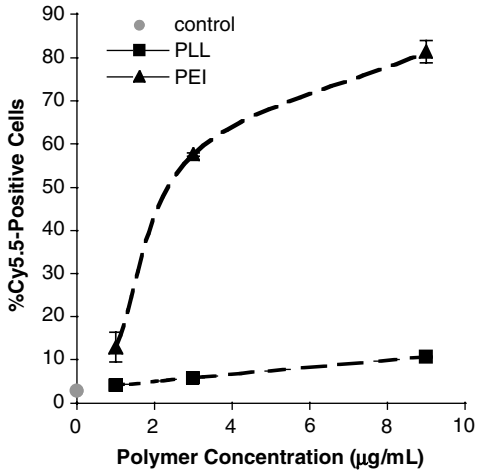


Fig. 5. Quantitative analysis of pEGFP-N2 uptake by flow cytometry. The Cy5.5-labeled pEGFP-N2 was used for complex formation with PLL and PEI at the indicated concentrations and cellular uptake of the plasmid was determined after 24 h. The control refers to BMSC incubated with the pEGFP-N2 alone without any polymer. The plasmid uptake was dependent on the polymer concentration, but PEI was more effective in the intracellular delivery of the plasmid. Cell-associated pEGFP-N2 was significantly higher ($p < 0.05$) for PEI as compared to PLL at all polymer concentrations tested.

3.4. Transfection efficiency by PLL and PEI

The expression of pEGFP-N2 was initially evaluated by epifluorescent microscopy. It was possible to observe EGFP-positive cells when pEGFP-N2 was delivered with PEI or PLL, in contrast to BMSC incubated with pEGFP-N2 alone (not shown). However, due to relatively weak nature of autofluorescence and its rapid decay, quantitation of EGFP-positive cells was considered unreliable in this setup. Alternatively, the transfection efficiency was assessed by flow cytometry. The BMSC was initially exposed to the plasmid DNA/polymer particles for 48 h. A relatively low percentage of cells (<4%) displayed EGFP expression initially (Fig. 6A), but after 1 week, a clear difference between the PEI and PLL-mediated delivery was evident at 3 and 9 μg/mL ($p < 0.02$), the PEI enabling a more effective EGFP expression as compared to PLL (Fig. 6A). Direct cell counts, however, indicated significant toxic effects on the cells by both polymers; Whereas DNA-exposed BMSC (control) cells displayed 2.4-fold increase in cell numbers from day 1 to 7, cells exposed to the polymer/pEGFP-N2 complexes did not exhibit cell growth during the 1-week period (Fig. 6B). By day 7, BMSC exposed to polymer/pEGFP-N2 complexes had lower cell counts than the day 1 cell counts (Fig. 6B), indicating significant impediment of the cell growth during the 1-week analysis period.

The transfection time was then reduced in a subsequent experiment to 8 h to minimize polymer effects on cell growth. As in 48-h exposure, BMSC exposed to polymer/pEGFP-N2 complexes similarly gave a relatively low transfection efficiency (<4%) on day 1. By day 7, 10–11% of BMSC were transformed with PLL-mediated delivery irrespective of the PLL concentration, whereas PEI resulted in 21–42% BMSC transfection depending on the concentration used (Fig. 6C). Due to large variation in EGFP expressed (given by relative fluorescence from flow cytometry) among the BMSC, it was not possible to correlate the level of GFP expression with the percentage of GFP-positive BMSC (not shown). This observation was in accordance with the previous studies utilizing linear PEI on cancer cell lines [34]. Unlike the 48-h transfection, a significant cell growth was seen with the 8-h transfections (Fig. 6D), indicating better tolerability of the complexes with the shorter exposure time. The only exception to this was (i) the BMSC exposed to highest concentration of PEI and PLL (9 μg/mL), which did not exhibit cell growth during the 7-day study period, and (ii) the BMSC exposed to highest concentration of PLL (9 μg/mL), where the increase in cell number did not reach a significantly higher value. This was indicative of the toxic nature of both polymers on the cells, a fact well appreciated and lead to extensive modification of the polymers to improve its compatibility with cells without affecting its transfection efficiency. Based on the plasmid delivery results (see Fig. 5), it appears that better effectiveness of the PEI was due to higher intracellular DNA delivery. It is not possible to rule out other beneficial effects of PEI, such as facilitation of endosomal escape [35].

The results presented in this study further confirm the feasibility of using polymeric carriers for transfection of bone marrow-derived cells, in addition to liposomal [36] and other lipid based carriers [37]. Effective expression was obtained with the latter carriers, but it is not possible to compare the current results with the lipid-based carriers from independent studies, since the percentage of cells transfected were not reported in the latter studies. As compared to some viral-based carriers, the polymeric-carriers explored in this study displayed an effectiveness that was at the lower end of the performance spectrum (especially with PLL), but the performance of PEI was considered reasonably successful; under conditions that allowed cell growth, transgene expression was sustained in ~20% of the cells for a 7-day period. This could be sufficient for applications that require transient expression, for example, when one needs to deliver osteogenic proteins with BMSC to initiate osteoinductive bone repair [36]. More studies for the duration of transgene expression will need to be performed to determine the potential of polymeric carriers for long-term transgene expression, which is necessary for diseases requiring constitutive gene expression. Towards this goal, two specific limitations suggested by this study were (i) the relatively low nuclear localization of the internalized plasmid (as compared to the nuclear localization of polymeric carriers), and (ii) undesirable effects of BMSC growth following polyplex treatment of the cells. To address the former limitation, one might need to engineer the polymers not to release their plasmid payload before reaching the nucleus. To address the latter limitation, more cell-compatible polymers will be required, perhaps by low-

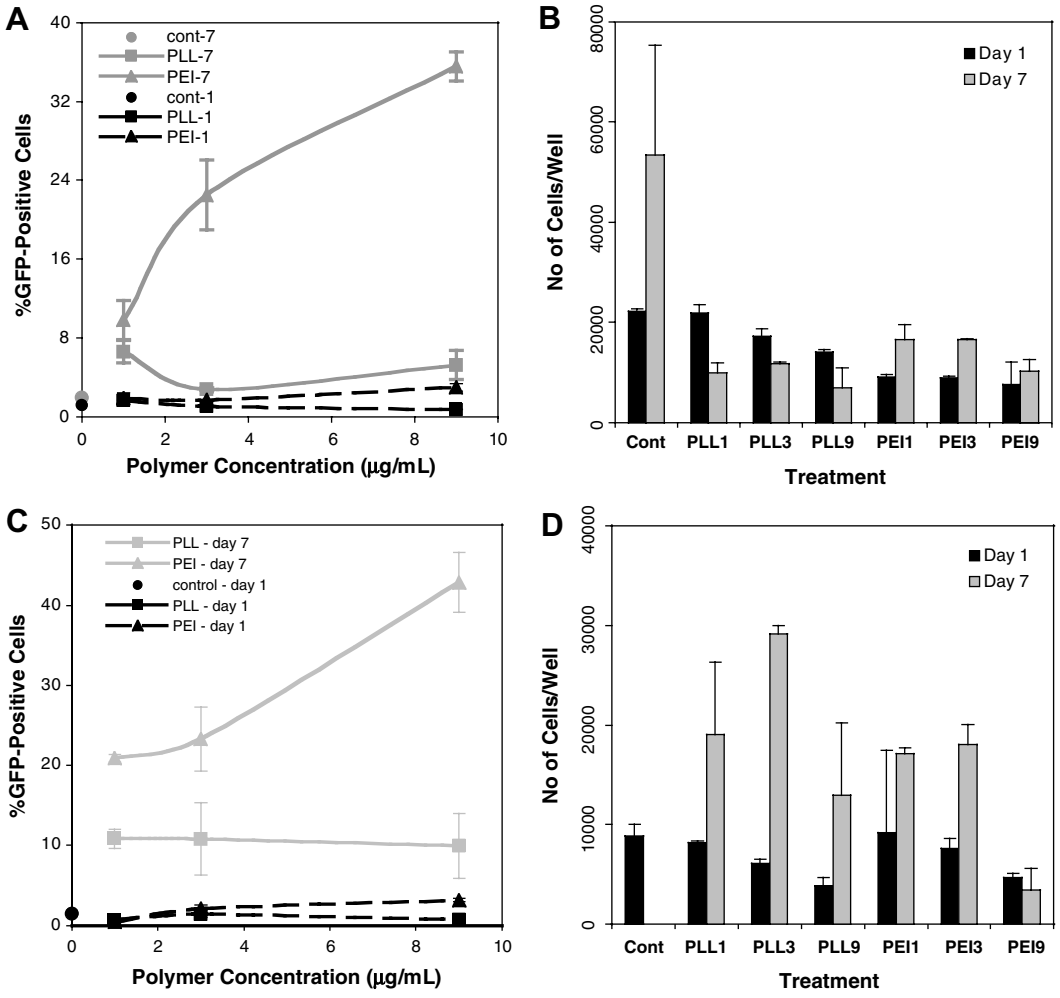


Fig. 6. Transfection of BMSC with pEGFP-N2/polymer complexes after 48-h (A and B) and 8-h (C and D) incubation of the complexes with the BMSC. (A) The BMSC was exposed to 3 μg pEGFP-N2 without any polymers (control), or the same amount of plasmid complexed with the indicated concentrations of the polymers. A relatively low percentage of BMSC was EGFP-positive after 1 day (black symbols and lines), after which PEI gave a robust transfection efficiency as a function of polymer concentration on day 7 (grey symbols and lines). (B) There was no significant increase in cell numbers in transfected wells after 1 week of culture, except PEI3 group ($p < 0.0001$). (C) As in 48-h transfection, a relatively low percentage of BMSC was EGFP-positive cells after 1 days, after which PEI gave a robust transfection efficiency as a function of polymer concentration used. PLL was less effective for EGFP expression. (D) Except the highest polymer concentrations ($p > 0.45$ and $p > 0.15$ for PEI and PLL, respectively), there was a significant cell growth in 8-h treated cells, indicating better compatibility of the complexes with the cells at the reduced exposure time.

ering their cationic charge density that appears to be the basis of the polymers' toxicity [38]. Given its more effective internalization and its ability to deliver the plasmid cargo intracellularly, PEI might be a more desirable option when future attempts to overcome these limitations for clinically effective modification of BMSC.

Acknowledgements

This project was financially support by an operating grant from the Canadian Institutes of Health Research (CIHR), and a discovery grant from Natural Sciences and Engineering Research Council of Canada (NSERC). We thank Basak Acan-Clements for preparing the pEGFP-N2 for this study and Weinan Hu for generating the AFM pictures. Expert technical help of Dorothy Rutkowski with flow cytometry (Department of Medical Microbiology and Immunology, University of Alberta) is appreciated.

References

[1] A. Van Damme, T. Vanden Driessche, D. Collen, M.K. Chuah, Bone marrow stromal cells as targets for gene therapy, Curr. Gene Ther. 2 (2002) 195–209.
[2] P.H. Krebsbach, K. Zhang, A.K. Malik, K. Kurachi, Bone marrow stromal cells as a genetic platform for systematic delivery of therapeutic proteins in vivo: human factor IX model, J. Gene Med. 5 (2003) 11–17.
[3] M. Takagi, Cell processing engineering for ex-vivo expansion of hematopoietic cells, J. Biosci. Bioeng. 99 (2005) 189–196.
[4] E.L. Herzog, L. Chai, D.S. Krause, Plasticity of marrow-derived stem cells, Blood 102 (2003) 3483–3493.
[5] J.M. Zimmet, J.M. Hare, Emerging role for bone marrow derived mesenchymal stem cells in myocardial regenerative therapy, Basic Res. Cardiol. 100 (2005) 471–481.

[6] N. Eliopoulos, A. Al-Khaldi, C.M. Beausejour, R.L. Momparler, L.F. Momparler, J. Galipeau, Human cytidine deaminase as an ex vivo drug selectable marker in gene-modified primary bone marrow stromal cells, Gene Ther. 9 (2002) 452–462.
[7] E.J. Schwarz, R.L. Reger, G.M. Alexander, R. Class, S.A. Azizi, D.J. Prockop, Rat marrow stromal cells rapidly transduced with a self-inactivating retrovirus synthesize L-DOPA in vitro, Gene Ther. 8 (2001) 1214–1223.
[8] A. Kume, M. Hashiyama, T. Suda, K. Ozawa, Green fluorescent protein as a selectable marker of retrovirally transduced hematopoietic progenitors, Stem Cells 17 (1999) 226–232.
[9] T. Relander, C. Fahlman, S. Karlsson, J. Richter, Low level of gene transfer to and engraftment of murine bone marrow cells from long-term bone marrow cultures, Exp. Hematol. 28 (2000) 373–381.
[10] R. Pawliuk, T. Bachelot, H. Raftopoulos, C. Kalberer, R.K. Humphries, A. Bank, P. Leboulch, Retroviral vectors aimed at the gene therapy of human beta-globin gene disorders, Ann. N.Y. Acad. Sci. 850 (1998) 151–162.
[11] J.C. Marx, J.A. Allay, D.A. Persons, S.A. Nooner, P.W. Hargrove, P.F. Kelly, E.F. Vanin, E.M. Horwitz, High-efficiency transduction and long-term gene expression with a murine stem cell retroviral vector encoding the green fluorescent protein in human marrow stromal cells, Hum. Gene Ther. 10 (1999) 1163–1173.
[12] J.D. Mosca, J.K. Hendricks, D. Buyaner, J. Davis-Sproul, L.C. Chuang, M.K. Majumdar, R. Chopra, F. Barry, M. Murphy, M.A. Thiede, U. Junker, R.J. Rigg, S.P. Forestell, E. Bohnlein, R. Storb, B.M. Sandmaier, Mesenchymal stem cells as vehicles for gene delivery, Clin. Orthop. Relat. Res. S379 (2000) S71–S90.
[13] P. Kurre, P. Anandakumar, H.P. Kiem, Rapid 1-hour transduction of whole bone marrow leads to long-term repopulation of murine recipients with lentivirus-modified hematopoietic stem cells, Gene Ther. 13 (2006) 369–373.
[14] Y. Deng, X. Guo, Q. Yuan, S. Li, Efficiency of adenoviral vector mediated CTLA4Ig gene delivery into mesenchymal stem cells, Chin. Med. J. (Engl.) 116 (2003) 1649–1654.
[15] B.A. Clements, J. Bai, C. Kucharski, L.L. Farrell, A. Lavasanifar, B. Ritchie, A. Ghahary, H. Uludag, RGD conjugation to polyethylenimine does not improve DNA delivery to bone marrow stromal cells, Biomacromolecules 7 (2006) 1481–1488.
[16] U. Lakshmipathy, B. Pelacho, K. Sudo, J.L. Linehan, E. Coucouvanis, D.S. Kaufman, C.M. Verfaillie, Efficient transfection of embryonic and adult stem cells, Stem Cells 22 (2004) 531–543.
[17] S.W. Kim, T. Ogawa, Y. Tabata, I. Nishimura, Efficacy and cytotoxicity of cationic-agent-mediated nonviral gene transfer into osteoblasts, J. Biomed. Mat. Res. 71 (2004) 308–315.
[18] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. USA 92 (1995) 7297–7301.
[19] G.Y. Wu, C.H. Wu, Receptor-mediated in vitro gene transformation by a soluble DNA carrier system, J. Biol. Chem. 262 (1987) 4429–4432, Erratum in: J. Biol. Chem. 262 (1988) 588.
[20] M. Varkey, C. Kucharski, T. Haque, W. Sebald, H. Uludag, In vitro osteogenic response of rat bone marrow cells to bFGF and BMP-2 treatments, Clin. Orthop. Rel. Res. 443 (2006) 113–123.
[21] T. Haque, H. Uludag, R.F. Zernicke, S.R. Winn, W. Sebald, Bone marrow cells from normal and ovariectomized rats respond differently to basic fibroblast growth factor and bone morphogenetic protein-2 treatment in vitro, Tissue Eng. 11 (2005) 634–644.
[22] J. Bai, B. Acan, A. Ghahary, B. Ritchie, V. Somayaji, H. Uludag, Polyethyleneimine/arginine-glycine-aspartic Acid conjugates prepared by *N*-succinimidyl 3-(2-pyridyldithio)propionate: an investigation of peptide coupling and conjugate stability, J. Polym. Sci. Polym. Chem. 42 (2004) 6143–6156.
[23] S. Grosse, Y. Aron, G. Thevenot, D. Francois, M. Monsigny, I. Fajac, Potocytosis and cellular exit of complexes as cellular pathways for gene delivery by polycations, J. Gene Med. 7 (2005) 1275–1286.
[24] B.R. Twaites, C. De Las Heras Alarcon, D. Cunliffe, M. Lavigne, S. Pennadam, J.R. Smith, D.C. Gorecki, C. Alexander, Thermo and pH responsive polymers as gene delivery vectors: effect of polymer architecture on DNA complexation in vitro, J. Control. Rel. 97 (2004) 551–566.
[25] H. Petersen, K. Kunath, A.L. Martin, S. Stolnik, C.J. Roberts, M.C. Davies, T. Kissel, Star-shaped poly(ethylene glycol)-block-polyethlenimine copolymers enhance DNA condensation of low molecular weight polyethylenimines, Biomacromolecules 3 (2002) 926–936.
[26] T. Okuda, S. Kidoki, M. Ohsaki, Y. Koyama, K. Yoshikawa, T. Niidome, H. Aoyagi, Time-dependent complex formation of dendritic poly(L-lysine) with plasmid DNA correlation with in vitro transfection efficiencies, Org. Biomol. Chem. 1 (2003) 1270–1273.
[27] C.-K. Chan, T. Senden, D.A. Jans, Supramolecular structure and nuclear targeting efficiency determine the enhancement of transfection by modified polylysines, Gene Ther. 7 (2000) 1690–1697.
[28] K. Miyata, Y. Kakizawa, N. Nishiyama, A. Harada, Y. Yamasaki, H. Koyama, K. Kataoka, Block catiomer polyplexes with regulated densities of charge and disulfide cross-linking directed to enhance gene expression, J. Am. Chem. Soc. 126 (2004) 2355–2361.
[29] K.H. Bremner, L.W. Seymour, A. Logan, M.L. Read, Factors influencing the ability of nuclear localization sequence peptides to enhance nonviral gene delivery, Bioconj. Chem. 15 (2004) 152–161.
[30] J.P. Clamme, G. Krishnamoorthy, Y. Mely, Intracellular dynamics of the gene delivery vehicle polyethylenimine during transfection: investigation by two-photon fluorescence correlation spectroscopy, Biochim. Biophy. Acta – Biomembr. 1617 (2003) 52–61.
[31] W.T. Godbey, K.K. Wu, A.G. Mikos, Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery, Proc. Nat. Acad. Sci. USA 96 (1999) 5177–5181.
[32] S.S. Talsma, J.E. Babensee, N. Murthy, I.R. Williams, Development and in vitro validation of a targeted delivery vehicle for DNA vaccines, J. Control. Rel. 112 (2006) 271–279.
[33] S. Grosse, Y. Aron, I. Honore, G. Thevenot, C. Danel, A.C. Roche, M. Monsigny, I. Fajac, Lactosylated polyethylenimine for gene transfer into airway epithelial cells: role of the sugar moiety in cell delivery and intracellular trafficking of the complexes, J. Gene Med. 6 (2004) 345–356.
[34] A. Ndoye, J.L. Merlin, A. Leroux, G. Dolivet, P. Erbacher, J.P. Behr, K. Berg, F. Guillemin, Enhanced gene transfer and cell death following p53 gene transfer using photochemical internalisation of glucosylated PEI–DNA complexes, J. Gene Med. 6 (2004) 884–894.
[35] A.M. Funhoff, C.F. van Nostrum, G.A. Koning, N.M. Schuurmans-Nieuwenbroek, D.J. Crommelin, W.E. Hennink, Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low pH, Biomacromolecules 5 (2004) 32–39.
[36] J. Park, J. Ries, K. Gelse, F. Kloss, K. von der Mark, J. Wiltfang, F.W. Neukam, H. Schneider, Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes, Gene Ther. 10 (2003) 1089–1098.
[37] R.R. Pochampally, E.M. Horwitz, C.M. DiGirolamo, D.S. Stokes, D.J. Prockop, Correction of a mineralization defect by overexpression of a wild-type cDNA for COL1A1 in marrow stromal cells (MSCs) from a patient with osteogenesis imperfecta: a strategy for rescuing mutations that produce dominant-negative protein defects, Gene Ther. 12 (2005) 1119–1125.
[38] D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis, Biomaterials 24 (2003) 1121–1131.