Surface modified poly(β amino ester)-containing nanoparticles for plasmid DNA delivery

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ABSTRACT

The use of biodegradable polymers provides a potentially safe and effective alternative to viral and liposomal vectors for the delivery of plasmid DNA to cells for gene therapy applications. In this work we describe the formulation of a novel nanoparticle (NP) system containing a blend of poly(lactic-co-glycolic acid) and a representative poly(beta-amino) ester (PLGA and PBAE respectively) for use as gene delivery vehicles. Particles of different weight/weight (wt/wt) ratios of the two polymers were characterized for size, morphology, plasmid DNA (pDNA) loading and surface charge. NPs containing PBAE were more effective at cellular internalization and transfection (COS-7 and CFBE41o−) than NPs lacking the PBAE polymer. However, along with these delivery benefits, PBAE exhibited cytotoxic effects that presented an engineering challenge. Surface coating of these blended particles with the cell-penetrating peptides (CPPs) mTAT, bPrPp and MPG via a PEGylated phospholipid linker (DSPE-PEG2000) resulted in NPs that reduced surface charge and cellular toxicity to levels comparable with NPs formulated with only PLGA. Additionally, these coated nanoparticles showed an improvement in pDNA loading, intracellular uptake and transfection efficiency, when compared to NPs lacking the surface coating. Although all particles with a CPP coating outperformed unmodified NPs, respectively, bPrPp and MPG coating resulted in 3 and 4.5× more pDNA loading than unmodified particles and approximately an order of magnitude improvement on transfection efficiency in CFBE41o− cells. These results demonstrate that surface-modified PBAE containing NPs are a highly effective and minimally toxic platform for pDNA delivery.

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1. Introduction

Biodegradable polymers are interesting alternatives to viral and liposomal vectors for plasmid DNA (pDNA) delivery. Several recent studies have examined pDNA delivery by degradable nanoparticles (NPs) formed from poly(lactic-co-glycolic acid) (PLGA), which was selected due to its biodegradability, ability to encapsulate and protect nucleic acid payloads from enzymatic degradation and its successful use in other drug delivery applications [1]. However, these PLGA formulations do not yield high enough pDNA expression levels necessary for clinical efficacy [2–5]. As a result, attention has shifted to other polymer systems to improve gene delivery and transfection efficiency, but no other polymer has the advantages of PLGA with respect to biocompatibility and FDA approval.

Poly(beta-amino) esters (PBAEs) are degradable, cationic polymers synthesized by conjugate (Michael-like) addition of bifunctional amines to diacrylate esters [6]. PBAEs appear to have properties that make them efficient vectors for gene delivery. These cationic polymers are able to condense negatively charged pDNA, induce cellular uptake, and buffer the low pH environment of endosomes leading to DNA escape [6,7]. PBAEs have the ability to form hybrid particles with other polymers, which allows for production of solid, stable and storable particles. For example, blending cationic PBAE with PLGA produced highly loaded pDNA microparticles. The addition of PBAE to PLGA resulted in an increase in gene transfection in vitro and induced antigen-specific tumor rejection in a murine model [8,9]. Although the blending of PBAE and PLGA has been explored for micron-sized particles aimed at phagocytic cells, no one has yet to explore the potential of this combination of polymers at submicron scales. In addition, although it is clear that surface modification of nanoparticles is an important element in the design of materials for drug delivery, there are no prior reports on the effectiveness of surface modification to improve delivery by PBAE/PLGA blended materials.

The efficiency of nanoparticle delivery systems can also be improved by the attachment of functional ligands to the NP surface. Potential ligands include, but are not limited to, small molecules, cell-penetrating peptides (CPPs), targeting peptides, antibodies or aptamers [10–13]. Attachment of these moieties serves a variety of different functions; such as inducing intracellular uptake, endosome disruption, and

delivery of the plasmid payload to the nucleus. There have been numerous methods employed to tether ligands to the particle surface. One approach is direct covalent attachment to the functional groups on PLGA NPs [5,14]. Another approach utilizes amphiphilic conjugates like avidin palmitate to secure biotinylated ligands to the NP surface [15,16]. This approach produces particles with enhanced uptake into cells, but reduced pDNA release and gene transfection, which is likely due to the surface modification occluding pDNA release [16]. In a similar approach, lipid-conjugated polyethylene glycol (PEG) is used as a multivalent linker of penetratin, a CPP, or folate [17]. These methods can be combined to tune particle function and efficacy.

In the present study we synthesized hybrid PBAE/PLGA NPs and optimized them for uptake and transfection of cystic fibrosis (CF) affected bronchiolar epithelial cells. After the initial optimization for PBAE wt/wt content, we surface modified these PBAE/PLGA NPs with phospholipid-PEG-CPP conjugates, and optimized these multifunctional particles to enhance NP uptake and transfection efficiency, while attenuating toxicity. These results provide insight into the design of PLGA-based NP formulations that could prove useful in the treatment of CF and other genetic disorders.

2. Materials and methods

2.1. Materials

Poly(D,L lactic-co-glycolic acid), 50:50 with inherent viscosity 0.95–1.20 dl/g, was purchased from DURECT Corporation (Birmingham, AL). Poly(beta amino ester) (PBAE) was synthesized by a Michael addition reaction of 1,4-butanediol diacrylate (Alfa Aesar Organics, Ward Hill, MA) and 4,4′-trimethylenedipiperidine (Sigma, Milwaukee, WI) as previously reported [6]. DSPE-PEG(2000)-maleimide was purchased from Avanti Polar Lipids (Alabaster, AL). Plasmid pGL4.13 DNA encoding firefly luciferase was purchased from Promega (Madison, WI), and produced at a scale for NP synthesis by transformation into competent DH5α cells, isolation with a Qiagen Giga Prep kit, and purification by ethanol precipitation.

2.2. Cells

COS-7 cells were obtained from ATCC (Manassas, VA) and cultured in α-Mem medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. IB3 cells were grown in DMEM (Gibco) supplemented with 10% FBS. CFBE41o− (CFBE) cells were grown in LHC-8 medium (Gibco) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/fungizone (anti-anti), 0.08 mg/ml tobramycin (Sigma), 0.04 mg/ml gentamicin (Sigma), and 0.002% Baytril (Bayer HealthCare AG)[18].

2.3. Synthesis of DSPE-PEG-peptide conjugates

CPPs were covalently linked to DSPE-PEG-maleimide as previously reported [17]. Briefly, cysteine-flanked (at the N-terminus) CPP was dissolved in 50 μl of diH$_2$O. A reaction mixture consisting of 50 μl TCEP bond breaker (ThermoScientific), 400 μl of 100 mM HEPES and 10 mM EDTA reaction buffer at pH 7.0–7.4, and 50 μl of the peptide solution was allowed to react at room temperature for 1 h. The reduced peptide solution was then added to 3× molar excess of DSPE-PEG-maleimide in reaction buffer and incubated at room temperature on a rotisserie rotator overnight. The next day the solution was dialyzed in 1× PBS to remove by-products from the reaction and stored at 4 °C until use. Conjugation was verified using matrix assisted laser desorption/ionization (MALDI) (Supplemental Fig. 1) and further quantified by SDS-PAGE using Novex 16% Tricine Gel System (Life Technologies) and Coomassie Blue staining per the manufacturer's protocol; sample reduction was performed with DTT (Supplemental Fig. 4).

2.4. Nanoparticle formulation and fabrication

Nanoparticles were formulated using a modified double emulsion solvent evaporation technique [16,19]. Varying wt/wt ratios of PLGA and PBAE were added to the oil phase (in dichloromethane) to determine an optimal formulation. Luciferase coding plasmid DNA (pGL4.13, Promega) in 1× Tris EDTA buffer was added dropwise under vortex to the solvent–polymer blend solution. The solution was then sonicated on ice using a probe sonicator (Tekmar Company, Cincinnati, OH) to form the first water-in-oil emulsion. The first emulsion was rapidly added to a 5.0% aqueous solution of poly(vinyl alcohol) under vortex to form the second emulsion and again sonicated. The emulsion was then added to a stirring 0.3% PVA stabilizer solution and stirred overnight to allow for residual solvent evaporation. Nanoparticles were centrifuged (3×, 9500 rpm, 15 min) and washed in diH$_2$O to remove excess PVA prior to lyophilization (72 h). Dried nanoparticles were stored at −20 °C until use.

To make fluorescent nanoparticles, coumarin-6 (C6) was added to the polymer solution at a 0.2% wt:wt C6:polymer ratio. To make surface-modified particles, DSPE-PEG-CPP was added to the 5.0% PVA solution during formation of the second emulsion at a 5 nmol/mg ligand-to-polymer ratio. Non-tethered DSPE-PEG-peptide and unconjugated peptide were removed during wash steps.

2.5. Nanoparticle characterization

2.5.1. Scanning electron microscopy
Morphology of gold coated particles was analyzed using an XL-30 scanning electron microscope (FEI, Hillsboro, Oregon). ImageJ software analysis was used to determine particle diameter.

2.5.2. Controlled release and loading
Known quantities of nanoparticles were incubated at 37 °C in 1 ml of PBS on a rotating shaker. At various time points particles were pelleted (10,000 rpm, 15 min) and the supernatant was removed and stored for analysis. An equal volume of fresh PBS was added to replace the supernatant and particles were resuspended and returned to the shaker. This process continued for 7 days. At the end of 7 days, the remaining particle pellets were added to DCM and the DNA still remaining in the particles was extracted into PBS two times to determine total loading. Samples were analyzed for DNA content using a Pico Green assay (Invitrogen).

2.5.3. DNA integrity
DNA integrity was determined by dissolution of particles into DCM and extraction of DNA into PBS. Samples were run on 1% agarose gel next to unprocessed plasmid DNA. Densitometry analysis was performed using ImageJ analysis software (Supplemental Fig. 3).

2.5.4. Zeta potential and hydrodynamic radius
Zeta potentials were measured using a Zetasizer Nano ZS (Malvern) with diH$_2$O as a dispersant at pH 6.0.

2.6. In-vitro transfection studies

2.6.1. Luciferase expression
COS-7 or CFBE41o− cells were seeded in 48-well plates at a seeding density of 1.5×10^4 cells/well and allowed to adhere overnight. Cells were washed once in PBS and treated with fresh medium containing serum and various concentrations of nanoparticles (in mg particles/ml serum containing medium). As an experimental control, in some experiments, cells were treated with medium containing mixtures of DNA and peptides or DSPE-PEG-peptide conjugates (Supplemental Fig. 6). Twenty four hours after treatment, particles were aspirated from cells, and then cells were washed with PBS and incubated in fresh medium. Lipofectamine 2000 (Invitrogen) was used as a positive control at an

optimal dose of 1 μl Lipofectamine and 0.5 μg DNA/well. Seventy-two hours after treatment, cells were lysed with 1× GloLysis Buffer (Promega), freeze/thawed twice at −80 °C, and measured for luciferase activity with a GloMax 20/20 spectrophotometer (Promega). For signal normalization, samples were also analyzed for total protein content using a micro BCA assay (Pierce).

2.6.2. GFP expression

Cells were treated with NPs as in Section 2.6.1 except that cells were seeded in 12-well plates at a seeding density of 1.5×10^5 cells/well. After 72 h, cells were imaged using fluorescence microscopy and then prepared for analysis by flow cytometry to look for GFP expression.

2.7. In vitro cytotoxicity studies

Cells seeded in 96-well plates were treated with particles as described above. Twenty four hours after treatment, spent medium with particles was removed, washed once with PBS, and fresh medium and CellTiter-Blue reagent (Promega) were added. After 4 h of incubation (COS-7) or 8 h of incubation (CFBE), plates were spun at 1300 rpm to pellet any remaining particles and the supernatant was transferred to a new black well, clear bottom plates. Fluorescence was measured at 562 nm according to the manufacturer's instruction.

2.8. Fluorescent particle uptake

2.8.1. Confocal microscopy

CFBE cells (1×10^4) were seeded in 8-well Lab Tek chamber slides and allowed to adhere overnight. Cells were treated with C6-loaded nanoparticles (as above Section 2.6.1) at a concentration of 0.05 mg/ml. Three or twenty-four hours later, cells were washed thoroughly with PBS, permeabilized with 0.1% Triton X-100, and fixed in 4% paraformaldehyde. Samples were stained with Texas red phalloidin for actin and mounted in vectashield with DAPI. Images and z-stacks were taken using a Leica SP5 confocal microscope.

2.9. Statistical analysis

All data for in vitro experiments were collected in triplicate and reported as mean +/− standard deviation. A paired t-test with 95% confidence interval ($p < .05$) was performed to determine the statistical significance of the different particle formulations in various experiments.

3. Results

3.1. Formulation and characterization of nanoparticles

We attempted to formulate NPs comprised of a blend of PBAE and PLGA with diameters less than 200 nm (Fig. 1A and B). As noted in previous works [6,19], PBAE's physical characteristics required modifications to the double emulsion protocol commonly used for PLGA alone. Most notably, solvent evaporation continued overnight (instead of the normal 3 h period used for PLGA alone) to ensure complete evaporation of DCM. Additionally, particles were centrifuged at 9500 rpm, as opposed to 12,000 rpm because at higher spin speeds particles had a tendency to fuse together. With these modifications, we were able to produce spherical particles approximately 150 nm in diameter (Fig. 1C) for wt/wt ratios (PBAE/PLGA) ranging from 0 to 25% (NP-0, NP-5, NP-15 and NP-25 respectively). Above the 25% wt/wt ratio particles failed to form or were highly fused together when viewed under SEM (data not shown). Increased levels of DNA encapsulation directly correlated with increasing PBAE content in the particles, presumably due to electrostatic interactions between the PBAE and DNA (Table 1). Zeta potential analysis revealed a substantial negative charge for particles formed from PLGA alone (NP-0), while particles containing PBAE (NP-5, NP-15, NP-25) had positive zeta potentials, which increased in
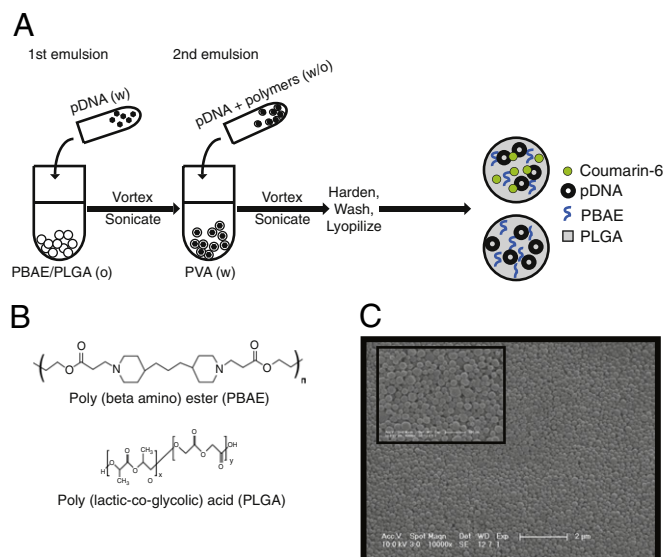

Fig. 1. Formulation and properties of PBAE/PLGA nanoparticles. (A) Nanoparticles with varying wt/wt ratios of PBAE and PLGA were formulated using a double emulsion/solvent evaporation technique. Particles were either encapsulated with plasmid DNA alone or co-encapsulated with coumarin-6 dye together with plasmid DNA. (B) Chemical structures of PBAE and PLGA polymers. (C) Representative scanning electron micrograph of 15% PBAE/PLGA blend nanoparticles.

magnitude as the fraction of PBAE increased and which we attribute to the incorporation of cationic PBAE.

3.2. In vitro DNA and coumarin-6 release

All particle formulations showed a burst release of their pDNA content within the first 24 h of incubation in PBS at physiologic pH (Fig. 2A). The NP-5 formulation released a significant fraction of its contents (68%) within the first 4 h, while the formulations with the higher PBAE content (NP-15 and NP-25) were sustained past 4 h (with only about 5% of its content released at that point) but before 24 h, by which time 89% and 77% respectively, were released. After the initial burst of pDNA, the release tapered significantly for all formulations. Overall amounts of plasmid DNA release over the first 7 days from particle formulations NP-5, 15 and 25 were 2, 5 and 7 times more, respectively, than the NP-0 formulation.
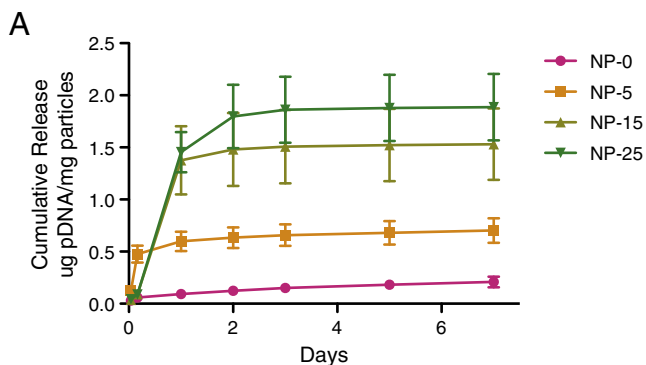
Particles formulated with C6 (a lipophilic dye) and pDNA were also tested for release of C6. Incubation of C6-NPs in PBS resulted in negligible C6 release: NP-0 particles released ~0.03% of the encapsulated C6 during a 24 h incubation in buffered saline and NP-15 released ~0.02% (Fig. 2B).

3.3. In vitro transfection of PBAE/PLGA blend nanoparticles

Particle formulations were tested for their ability to transfect cells in culture. We chose two cell lines to study: COS-7 cells were chosen as an easily transfectable, SV-40 transformed cell line and ΔF508 CFBE41o− cells were chosen as a relevant target cell line for therapy. COS-7 cells responded differently to particle treatment than did CFBE

Table 1
Characterization of nanoparticle preparations for mean size (diameter), loading of plasmid DNA (by extraction and analysis), and surface charge (zeta potential).

Formulation (wt.% PBAE)	Diameter (nm +/− SD)	Loading (μg/mg +/− SD)	Encaps. eff. (%)	Zeta potential (my +/− SD)
PLGA alone	160 +/− 77	0.32 +/− 0.09	3.2	−32 +/− 6.0
5%	149 +/− 53	0.76 +/− 0.13	7.6	9.91 +/− 5.0
15%	165 +/− 68	1.61 +/− 0.39	16.1	30.2 +/− 7.0
25%	137 +/− 67	2.12 +/− 0.38	21.2	29.53 +/− 14.5

Fig. 2. Release profile of different PBAE/PLGA blend NPs. (A) Release of plasmid DNA over a one week period. (B) Release of coumarin-6 after 24 h.

Formulation	C6 Released	% of Highest Theoretical Loading
NP-0	0.00061 +/- 0.00018	0.03
NP-15	0.00041 +/- 0.000075	0.02

cells, exhibiting higher luciferase expression in response to particle treatments (Fig. 3A and B). In all cases for both cell lines, lower particle doses (0.05 mg/ml or 0.5 mg/ml) produced the highest transfection efficiency. For lower dose treatments of NP-25 and NP-15 in COS-7 and CFBE cells, respectively, we saw a significant increase in transfection efficiency compared to the NP-0 treatment. This did not hold true for higher doses (1 mg/ml and 2 mg/ml). It is possible that the transfection enhancement produced by NP-15 and NP-25 over NP-0 at the lower doses is due primarily to the increased loading of pDNA within the PBAE containing NPs. We note, however, that at high doses of NP-0 particles (2 mg/ml), the amount of pDNA administered in culture is nearly equivalent to the amount of pDNA in the 0.05 mg/ml dose of NP-15 treatment; the transfection resulting from NP-0 was 100–1000 times less than the transfection resulting from NP-15 in both cell lines.

The optimal particle doses in each cell line produced luciferase expression at levels that were similar to Lipofectamine 2000 (Invitrogen) (i.e. within a factor of 10), but with less pDNA. For example, NP-15 at 0.5 mg/ml contained 0.2 μg/well pDNA and NP-25 at 0.05 mg/ml contained 0.013 μg/well, well below the 0.5 μg/well DNA that was applied with Lipofectamine.

Particle toxicity was evaluated by measuring cell viability after exposure to controlled doses of particles (Fig. 3C, D). In both cell lines there was a substantial increase in particle toxicity with increases in dose and PBAE content. NP-15 and NP-25 formulations produced considerable cell death in both COS-7 and CFBE cells, especially at high doses, but CFBE cells were more sensitive to particles at low doses than COS-7.

3.4. Fluorescent particle uptake by CFBE cells

Confocal microscopy was used to examine whether the enhanced transfection observed with the NP-15 vs. the NP-0 formulation was correlated with increased uptake of the particles. CFBE cells were exposed to NPs containing C6. A z-stack of a representative cell treated with NP-15 after 3 h (Fig. 4C, Supplemental Fig. 2) revealed a widespread particle association and internalization. This internalization increased after 24 h (Fig. 4D). In contrast, NP-0 particles showed a little cellular association and internalization after 3 h (Fig. 4A). Although internalized NP-0 particles were detected after 24 h (Fig. 4B), as revealed by rotating the z-stack, there were far fewer NPs compared with the NP-15 treatment.

3.5. Surface modified PBAE/PLGA blend nanoparticles

In prior work, we showed that a heterobifunctional construct consisting of PEG covalently linked to DSPE—a lipid that tethers PEG to the NP surface through interaction with the hydrophobic polymer matrix—can be used to coat the surface of PLGA NPs with PEG and PEG-ligand conjugates [17]. Here, we used that approach to add various CPPs to PBAE/PLGA NPs. To accomplish this, we first synthesized
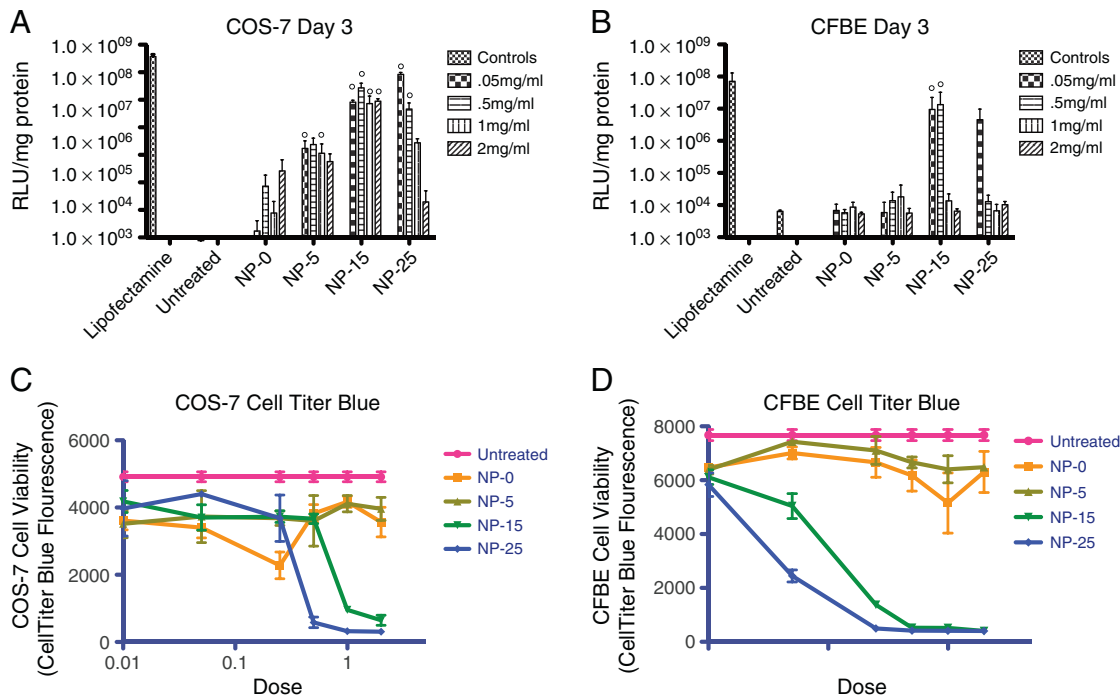

Fig. 3. Transfection of (A) COS-7 and (B) CFBE cells treated with PBAE/PLGA blend nanoparticles encapsulating pGL4.3 DNA and normalized to total protein content. Particle formulations contained PLGA and 0%, 5%, 15% and 25% PBAE blend at .05, .5, 1 and 2 mg/ml particle doses. Transfection efficiencies were compared to Lipofectamine 2000 (prepared according to the manufacturer's instructions) as a positive control and untreated cells as a negative control. Toxicity of PBAE/PLGA formulations was also evaluated in (C) COS-7 and (D) CFBE cells 24 h post-treatment using cell titer blue. A ° indicates statistically greater values than those of corresponding particles formulated with PLGA alone at the corresponding dose.
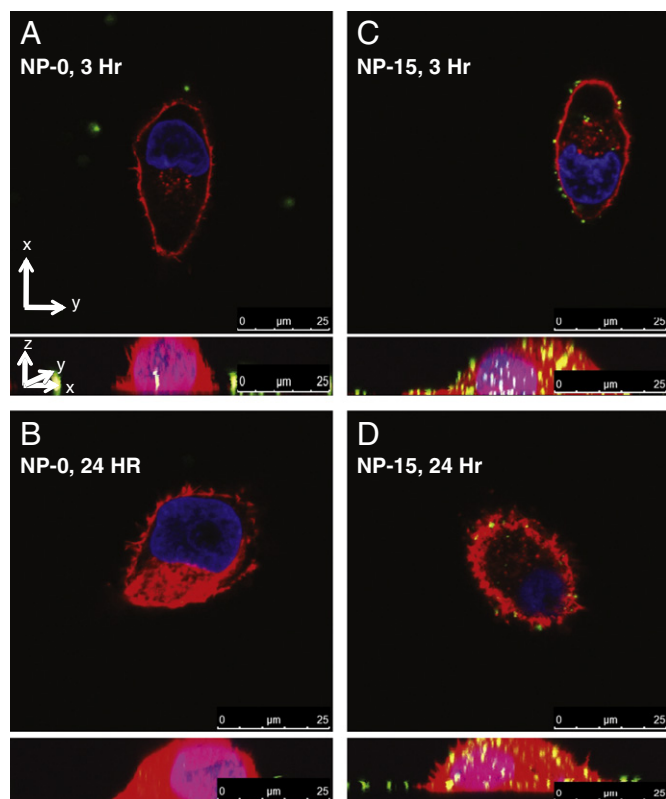
Fig. 4. Confocal microscopy of CFBE cells treated with coumarin-6 loaded NP-0 vs. NP-15 blend nanoparticles. (A) NP-0 at 3 h post-treatment, (B) NP-0 at 24 h post-treatment, (C) NP-15 at 3 h post-treatment and (D) NP-15 at 24 h post-treatment. Top images represent slices from the middle of a z-stack of a single CFBE cell. Bottom images are projections in the z-axis created from a z-stack of the same CFBE cell displayed in the top image. Cells were treated with coumarin-6 loaded nanoparticles (green), fixed and stained with DAPI (nucleus, blue), and Texas red phalloidin (actin, red).

DSPE-PEG-CPP conjugates (Supplemental Fig. 1); we chose three CPPs—mTAT, MPG, and bPrPp (Table 2)—all of which have been shown to enhance nucleic acid delivery [20–23]. We then synthesized NP-15 particles with DSPE-PEG-CPP coatings. All particles with the surface coating were morphologically similar to those without coatings (Fig. 5A). Particles with the surface coating also loaded pDNA more efficiently than those without modification; for example, DSPE-PEG-bPrPp and DSPE-PEG-MPG coated particles loaded 3 and 4.5 times more pDNA than unmodified particles, respectively (Fig. 5C). When incubated in buffered saline, particles coated with DSPE-PEG-bPrPp and DSPE-PEG-MPG released pDNA content for a longer period than unmodified particles (Fig. 5B). Unmodified NP-15 particles released 95% of total pDNA content after a 48 h incubation time, while bPrPp and MPG coated NPs released 54 and 80% respectively of their total at that time point. In addition, particles with the DSPE-PEG-CPP coatings had 50 to 75% lower zeta potentials than their unmodified counterparts (Fig. 5C).

Table 2
Description of CPPs conjugated to DSPE-PEG for surface modification of PBAE/PLGA NPs.

Peptide name	Sequence	Reference	Origin
mTAT	HHHHRKKRRQRRRHHHHH	Yamano et al. [23]	HIV-1 (with histidine modification)
bPrPp	MVKSKIGSWILVLFVAMWS DVGLCKKRPKP	M. Magzoub et al. [21]	Bovine prion
MPG	GALFLGFLGAAGSTMGAWS QPKKKRKV	T. Endoh et al. [20]	Synthetic chimera: SV40 Lg T. Ant.＋HIV gb41 coat
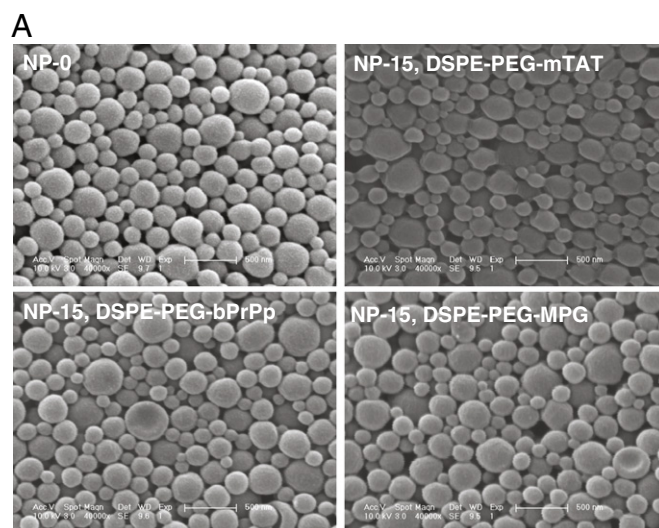
B

Release of pDNA from Surface Modified PBAE/PLGA Nanoparticles

Legend:
- NP-0
- NP-15
- NP-15, DSPE-PEG-mTAT
- NP-15, DSPE-PEG-bPrPp
- NP-15, DSPE-PEG-MPG
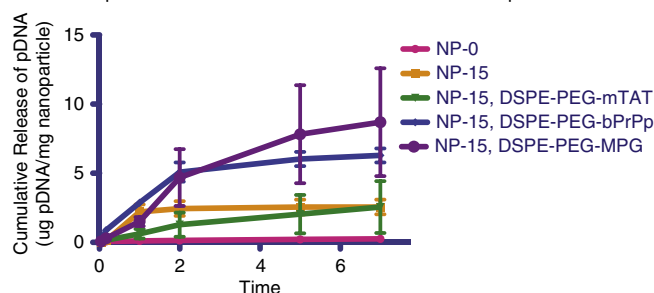
C

Formulation	Diameter (nm +/- SD)	Loading (ug/mg +/- SD)	Encaps. Eff. (%)	Zeta Potential (mV +/- SD)
N0-P	148 +/- 83	0.35 +/- 0.14	3.6	-32.0 +/- 6.1
N51-P	140 +/- 74	1.96 +/- 0.28	19.7	34.5 +/- 5.5
NP-15, DSPE-PEG-mTAT	158 +/- 66	2.77 +/- 1.95	27.8	16.8 +/- 4.4
NP-15, DSPE-PEG-bPrPp	180 +/- 72	6.48 +/- 0.52	64.9	9.1 +/- 4.3
NP-15, DSPE-PEG-MPG-NLS	178 +/- 72	8.96 +/- 3.95	89.7	13.4 +/- 4.9

Fig. 5. NP-15 particles surface modified with cell permeable peptides (A) SEM micrographs, (B) controlled release profile and (C) characterization of size, DNA loading and surface charge.

3.6. Surface modified PBAE/PLGA blend nanoparticles enhance particle uptake and gene expression

3.6.1. Particle uptake by CFBE cells

C6 loaded NP-15 particles with DSPE-PEG-CPP surface coatings were used to assess cellular association and uptake of NPs (Fig. 6). NPs with the DSPE-PEG-mTAT or DSPE-PEG-bPrPp showed increased uptake, as revealed by the presence of substantially more cell-associated fluorescence than observed in cells exposed to particles with no surface coating. The effect was most obvious with DSPE-PEG-bPrPp NPs, which showed the most substantial particle fluorescence, especially throughout the cytoplasm. MPG coated particles also showed enhanced uptake beyond uncoated NP-15 particles. This was confirmed with flow cytometry analysis in a similar CF cell line (Supplemental Fig. 5).

3.6.2. In vitro transfection and cytotoxicity

DSPE-PEG-CPP coated particles loaded with luciferase encoding pDNA were incubated with CFBE cells and tested for transfection 3 and 7 days post-NP treatment (Fig. 7A and B). Unlike unmodified particles, where luciferase expression was lower at the highest doses (in mg particle/ml) (Fig. 3), peptide-modified particles led to an increase in gene expression with increasing dose. The highest doses (1 and 2 mg/ml) of DSPE-PEG-bPrPp and DSPE-PEG-MPG
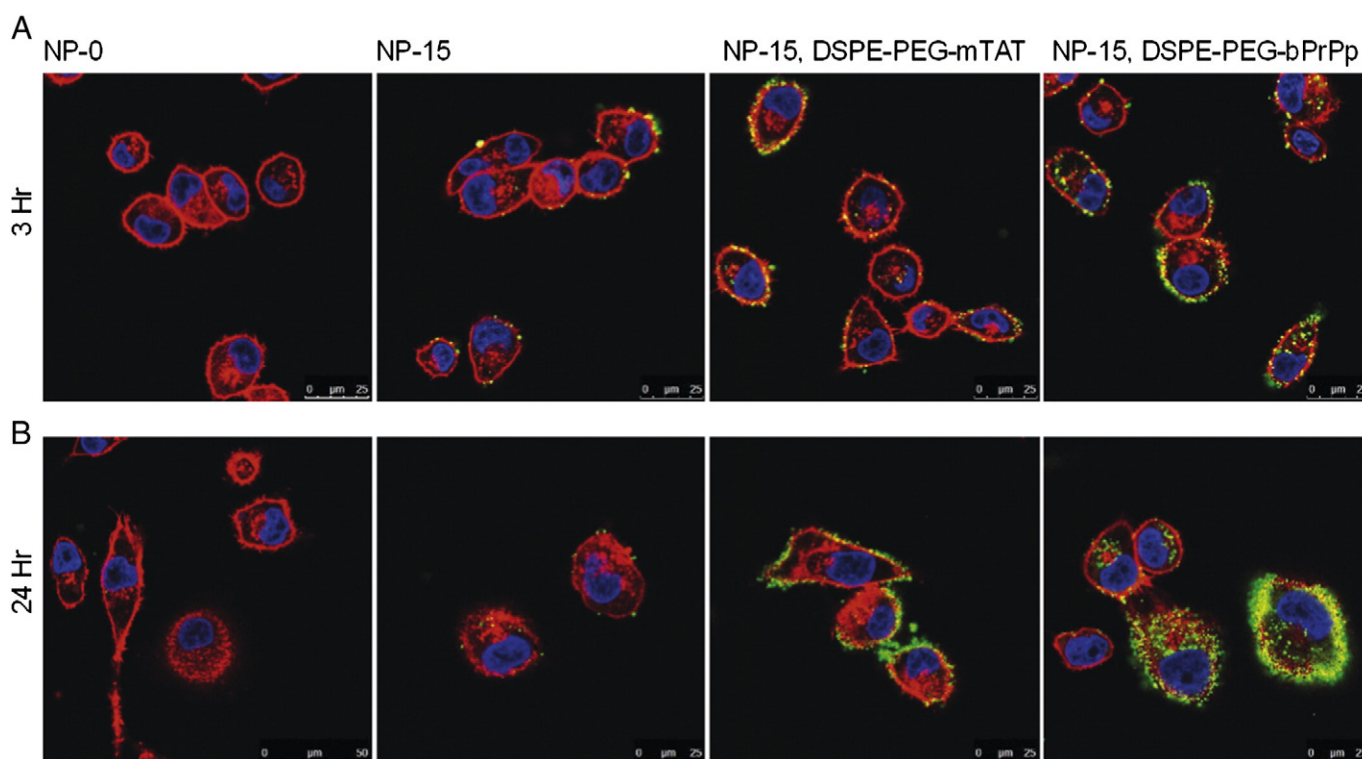
Fig. 6. Confocal microscopy of CFBE cells treated with coumarin-6 loaded NP-0, NP-15, and surface modified NP-15 nanoparticles at (A) 3 h and (B) 24 h post-treatment. Images were obtained from the middle of a z-stack encompassing the height of the cells. Cells were treated with coumarin-6 loaded nanoparticles (green), fixed and stained with DAPI (nucleus, blue), and Texas red phalloidin (actin, red). Zoom: 188 on machine objective: 63× (with immersion oil).

coated NPs resulted in significantly higher luciferase expression than the most effective doses (0.05 and 0.5 mg/ml) of unmodified NP-15 particles, at both 3 and 7 days after particle treatment. Additionally, for cells treated with particles modified with bPrPp and MPG, luciferase expression was on the same order of magnitude as Lipofectamine 2000-treated cells (although at the high particle doses, there was more pDNA/well in the Lipofectamine 2000 treated cells). Control experiments confirm that the transfection produced by DSPE-PEG-CPP nanoparticles was several orders of magnitude more efficient than the transfection produced by free peptide or DSPE-PEG-peptide conjugates (Supplemental Fig. 6).

FACS analysis was performed to determine the % of cells expressing protein in our most promising NP formulations (NP-15 modified with MPG and bPrPp) using GFP pDNA. Our results indicate that at 2 mg/ml particle dose MPG modified NP-15 transfected 3% of the cell population and bPrPp modified NP-15 transfected 5.5% of the CFBE cells (Supplemental Fig. 7).

We tested modified particles for their effect on cell viability (Fig. 7C). Cells exposed to particles with DSPE-PEG-CPP coatings showed minimally reduced viability compared to untreated controls, whereas unmodified NP-15 treatment caused significant toxicity at doses as low as 0.1 mg/ml.

4. Discussion

Lack of efficiency and/or safety of viral and liposomal vectors for the treatment of genetic disorders, such as CF, has prompted investigation into novel polymer-based systems for disease treatment. PLGA represents an FDA-approved, biocompatible material that has been extensively studied for gene delivery purposes but has also lacked efficiency as a gene delivery vehicle on its own [14,24,25]. This lack of efficient expression is probably due to the negative charge on PLGA

at physiologic pH, which leads to low DNA encapsulation and limited association with the negatively charged plasma membrane of cells. Consistent with this hypothesis, loading and gene expression can be enhanced by conjugating PLGA with cationic polymers, such as poly(L-lysine) but thus far only modest improvements in gene transfer effectiveness have been reported [5]. In this work we sought to further improve upon a PLGA based system by formulating PBAE/PLGA blend NPs similar to microparticle formulations previously developed for gene vaccines [8,9,19]. We found that we could form nanosized PBAE/PLGA blend NPs with average diameters less than 200 nm. We were able to create particles with up to 25%wt content of PBAE: at higher PBAE content, NPs failed to form or were highly fused together when viewed under SEM (data not shown). This finding is consistent with other reports [6] and the 25% maximum ratio is likely due to the physical properties of the PBAE. Regardless of this limitation, NPs containing PBAE could more efficiently load and release DNA in solution, as well as internalize into and transfect multiple cell types more effectively than NPs formulated with PLGA alone.

Nanoparticles formed with 15 or 25% PBAE content showed the greatest transfection capability in both fragile, disease-related CFBE41o− cells and more robust COS-7 cells respectively. However, although increasing PBAE content resulted in increased transfection efficiency, increasing particle dose while maintaining the same PBAE wt content showed a marked decrease in transfection efficiency in both cell lines. This result was surprising, as increasing dose also increases DNA payload, which should lead to increased, not reduced, gene expression. As a result of these findings we sought to examine the potential toxic effects of the NPs. CellTiter-Blue analysis indicated that increasing PBAE content, whether through increasing wt/wt content of PBAE within the particles, or via increasing NP dose, resulted in increased toxicity of the particles in both COS-7 and CFBE cells. COS-7 cells were more resistant to toxic effects of the NPs than were
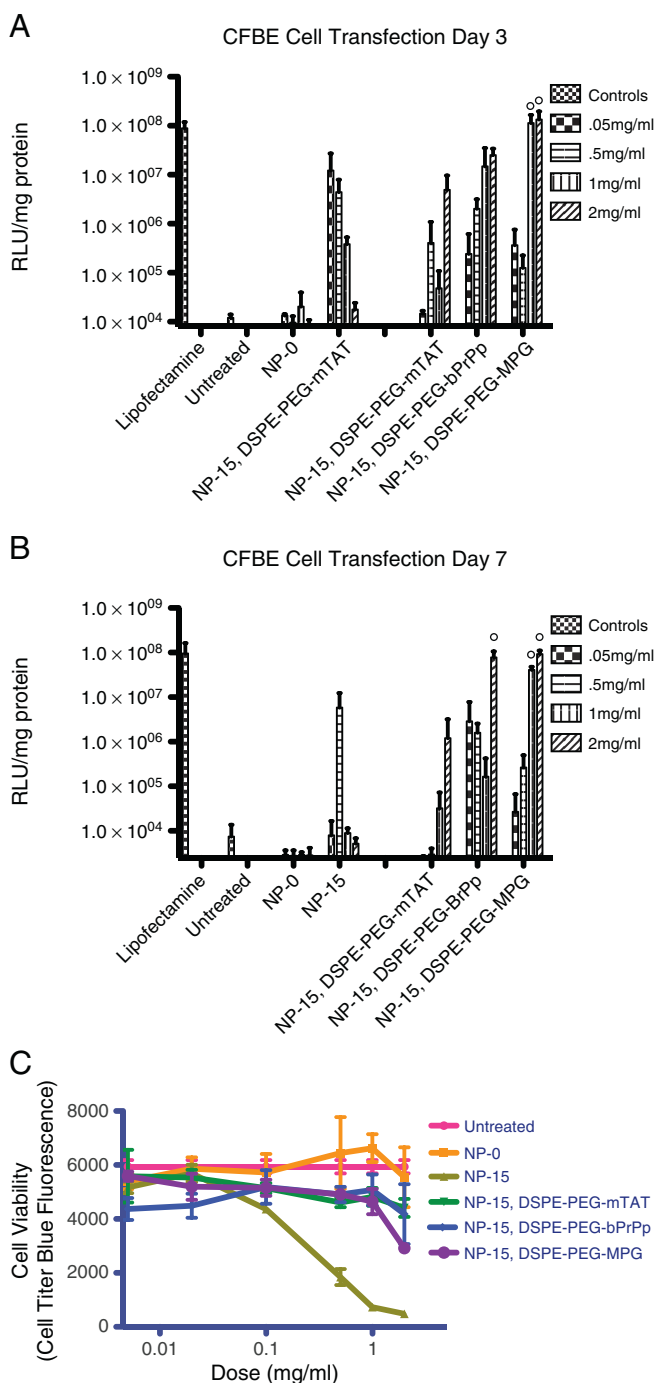
Fig. 7. Transfection of CFBE cells with surface modified NP-15 nanoparticles encapsulating pGL4.3 DNA (A) 3 and (B) 7 days after particle treatment and normalized to cellular protein content. Cells were treated with NP-0 and unmodified NP-15 nanoparticles as well as NP-15 particles that had the additional DSPE-PEG-peptide conjugates on the surface. Peptide conjugates were mTAT, bPrPp and MPG. Cells were treated with .05, 0.5, 1 and 2 mg/ml particle doses. (C) Particle toxicity was also evaluated using cell titer blue. A ° indicates that values are statistically greater (p<.05) than those of the best performing dose (.05 mg/ml for Day 3 and .5 mg/ml for Day 7) of NP-15 particles that have not been surface modified.

This is a problem for many cationic delivery vehicles — although the polymers are effective at improving DNA delivery and expression, the same mechanism that results in this improvement also causes toxicity [26]. Hence, we sought methods to reduce the toxicity of the formulations.

To further improve upon the efficiency and reduce the toxicity of this system, we formulated particles with a heterobifunctional DSPE-PEG-CPP coating. This amphiphilic molecule was designed to hydrophobically associate with the polymer matrix via the acyl chains in DSPE, and present a PEG-CPP moiety on the particle surface [17]. We believed PEG could serve a variety of functions such as to reduce aggregation and aid in diffusion of the particles [27,28], and shield the high surface charge imparted by the PBAE. Additionally the surface-bound PEG could be coupled to moieties that aid in intracellular uptake and endosomal escape, such as CPPs, to the NP surface [17,29,30]. We chose to add this surface modification to NP-15 particles because they showed the most promise as a transfection vehicle in CFBE41o− cells (Fig. 3B). After introducing this surface modification with a variety of different CPPs (Table 2), we characterized our surface-modified particles vs. the unmodified particles.

Our studies show that coating nanoparticles with DSPE-PEG-CPP conjugates provided many advantages. Conjugate coating increased the overall loading and release of pDNA compared to unmodified control particles (Fig. 5C). Additionally, particles with the DSPE-PEG-CPP coating had reduced zeta potentials (most likely due to the presence of PEG and an increase in DNA loading, both of which neutralized the positive charge of the PBAE) that we hypothesized led to an increase in cell viability similar to particles formulated with PLGA alone (Fig. 7C). The large reduction in surface charge due to the addition of the DSPE-PEG-CPP constructs could have resulted in reduced non-specific particle association and uptake in cells, but this was not observed. Rather, particles with the DSPE-PEG-CPP coating provided for enhanced intracellular uptake (Fig. 6) as confirmed by confocal microscopy of coumarin-6 loaded NPs. Lastly, particles with the DSPE-PEG-CPP coating were able to transfect cells more efficiently with increasing particle dose most likely due to the higher tolerance cells showed for these surface modified particles. At optimal doses (1–2 mg/ml), in two out of three DSPE-PEG-CPP coated particle groups (bPrPp and MPG), we saw a significant increase in the overall transfection compared with the highest transfection efficiency observed at any dose with uncoated particles.

The different CPPs we tested (mTAT, MPG, and bPrPp) resulted in different physical properties and transfection capabilities of the particles. Although all coated particle formulations resulted in a substantial decrease in zeta potential, mTAT exhibited only modest improvements on loading and transfection efficiency compared to unmodified particles, while the use of bPrPp and MPG resulted in ~3 and 4.5× improvements on loading and release and approximately an order of magnitude improvement on transfection efficiency. This is possibly due to the different mechanisms of action utilized by these different peptides. MPG and bPrPp, unlike mTAT, contain cell-penetrating and nuclear localization (NLS) motifs. The improved transfection observed with these two peptides could be the result of enhanced pDNA trafficking to the nucleus due to the presence of an NLS. Additionally, it is thought that the mechanism of uptake and endosomal escape for both bPrPp and MPG is lipid perturbation while mTAT is thought to enter cells via endocytosis pathways [20,21,31,32]. Another possible explanation for the discrepancy in performance between the different peptides is the amount of DSPE-PEG-CPP loading on the particle surface. Five nmol/mg DSPE-PEG-CPP conjugate was added to the second emulsion for incorporation onto the particle surface, however the 5 nmol/mg amount assumed 100% efficiency in DSPE-PEG-maleimide conjugation to cysteine flanked CPPs. Also, it is possible that different amino acid compositions load more or less favorably onto the particle surface due to the different properties inherent to those peptide sequences. Future studies will seek to better characterize DSPE-PEG-CPP loading onto the surface of particles and its effects on the system.

CFBE cells. This disparity in cytotoxicity (Fig. 3C and D) might help explain the enhanced efficiency of COS-7 cell transfection compared to CFBEs (Fig. 3A, B). These cytotoxic effects are likely due to the high positive surface charge imparted by the PBAE, which is apparent from the increased zeta potential with increasing PBAE content. We hypothesize that the increase in cytotoxic effects with increasing particle dose resulted in the observed reduction in transfection efficiency.

GENE DELIVERY

5. Conclusions

PLGA systems have been extensively studied for gene delivery but have yet to show the efficiency necessary for therapeutic applications. In this study we show that the delivery of pDNA from PLGA NPs can be significantly improved by rationally engineering traditional PLGA formulations. Our first modification was the addition of a second polymer, PBAE, to the matrix, which improved pDNA loading and release, but at the cost of added toxicity to the cells. Our second modification was the addition of a DSPE-PEG-CPP conjugate to the NP surface, which further enhanced DNA loading, release and transfection capabilities of the NPs and attenuated PBAE toxicity. This is the first report of a system in which CPP modifications of polymer nanoparticles have been found to be multifunctional. In this case, the addition of the CPP—using the phospholipid-PEG-conjugate—provides at least two functions: 1) increase in the loading of pDNA within the nanoparticles and 2) dramatic decrease in the cytotoxicity of the cationic nanoparticles with an increase in transfection efficiency. This system represents a novel platform for pDNA delivery with potential therapeutic uses in diseases such as CF.

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

References

[1] D. Luo, K. Woodrow-Mumford, N. Belcheva, W.M. Saltzman, Controlled DNA delivery systems, Pharm. Res. 16 (1999) 1300–1308.
[2] T. Wang, J.R. Upponi, V.P. Torchilin, Design of multifunctional non-viral gene vectors to overcome physiological barriers: dilemmas and strategies, Int. J. Pharm. 427 (1) (2012) 3–20.
[3] A.K. Jain, M. Das, N.K. Swarnakar, S. Jain, Engineered PLGA nanoparticles: an emerging delivery tool in cancer therapeutics, Crit. Rev. Ther. Drug Carrier Syst. 28 (2011) 1–45.
[4] M. Conese, F. Ascenzioni, A.C. Boyd, C. Coutelle, I. De Fino, S. De Smedt, J. Rejman, J. Rosenecker, D. Schindelhauer, B.J. Scholte, Gene and cell therapy for cystic fibrosis: from bench to bedside, J. Cyst. Fibros. 10 (Suppl. 2) (2011) S114–S128.
[5] J. Blum, W. Saltzman, High loading efficiency and tunable release of plasmid DNA encapsulated in submicron particles fabricated from PLGA conjugated with poly-ʟ-lysine, J. Control. Release 129 (2008) 66–72.
[6] D.M. Lynn, Degradable poly(B-amino esters): synthesis, characterization, and self-assembly with plasmid DNA, in: R. Langer (Ed.), J. Am. Chem. Soc., 2000, pp. 10761–10768.
[7] J. Green, R. Langer, D. Anderson, A combinatorial polymer library approach yields insight into nonviral gene delivery, Acc. Chem. Res. 41 (6) (2008) 749–759.
[8] S.R. Little, D.M. Lynn, Q. Ge, D.G. Anderson, S.V. Puram, J. Chen, H.N. Eisen, R. Langer, Poly-beta amino ester-containing microparticles enhance the activity of nonviral genetic vaccines, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 9534–9539.
[9] S.R. Little, D.M. Lynn, S.V. Puram, R. Langer, Formulation and characterization of poly(beta amino ester) microparticles for genetic vaccine delivery, J. Control. Release 107 (2005) 449–462.
[10] C. Yu, Y. Hu, J. Duan, W. Yuan, C. Wang, H. Xu, X.D. Yang, Novel aptamer-nanoparticle bioconjugates enhances delivery of anticancer drug to MUC1-positive cancer cells in vitro, PLoS One 6 (2011) e24077.
[11] Y. Cu, C.J. Booth, W.M. Saltzman, In vivo distribution of surface-modified PLGA nanoparticles following intravaginal delivery, J. Control. Release 156 (2011) 258–264.
[12] H. Nie, S.T. Khew, L.Y. Lee, K.L. Poh, Y.W. Tong, C.H. Wang, Lysine-based peptide-functionalized PLGA foams for controlled DNA delivery, J. Control. Release 138 (2009) 64–70.
[13] L.J. Cruz, P.J. Tacken, R. Fokkink, B. Joosten, M.C. Stuart, F. Albericio, R. Torensma, C.G. Figdor, Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN in vitro, J. Control. Release 144 (2010) 118–126.
[14] J.P. Bertram, S.M. Jay, S.R. Hynes, R. Robinson, J.M. Criscione, E.B. Lavik, Functionalized poly(lactic-co-glycolic acid) enhances drug delivery and provides chemical moieties for surface engineering while preserving biocompatibility, Acta Biomater. 5 (2009) 2860–2871.
[15] T. Fahmy, R. Samstein, C. Harness, W. Mark Saltzman, Surface modification of biodegradable polyesters with fatty acid conjugates for improved drug targeting, Biomaterials 26 (2005) 5727–5736.
[16] Y. Cu, C. LeMoëllic, M. Caplan, W. Saltzman, Ligand-modified gene carriers increased uptake in target cells but reduced DNA release and transfection efficiency, Nanomedicine 6 (2010) 334–343.
[17] C.J. Cheng, W.M. Saltzman, Enhanced siRNA delivery into cells by exploiting the synergy between targeting ligands and cell-penetrating peptides, Biomaterials 32 (2011) 6194–6203.
[18] D.C. Gruenert, M. Willems, J.J. Cassiman, R.A. Frizzell, Established cell lines used in cystic fibrosis research, J. Cyst. Fibros. 3 (Suppl. 2) (2004) 191–196.
[19] D.N. Nguyen, J.J. Green, J.M. Chan, R. Langer, D.G. Anderson, Polymeric Materials for Gene Delivery and DNA Vaccination, Adv. Mater. 21 (2009) 847–867.
[20] T. Endoh, T. Ohtsuki, Cellular siRNA delivery using cell-penetrating peptides modified for endosomal escape, Adv. Drug Deliv. Rev. 61 (2009) 704–709.
[21] M. Magzoub, S. Sandgren, P. Lundberg, K. Oglecka, J. Lilja, A. Wittrup, L.E. Göran Eriksson, U. Langel, M. Belting, A. Gräslund, N-terminal peptides from unprocessed prion proteins enter cells by macropinocytosis, Biochem. Biophys. Res. Commun. 348 (2006) 379–385.
[22] J. Zhou, T.R. Patel, M. Fu, J.P. Bertram, W.M. Saltzman, Octa-functional PLGA nanoparticles for targeted and efficient siRNA delivery to tumors, Biomaterials 33 (2012) 583–591.
[23] S. Yamano, J. Dai, C. Yuvienco, S. Khapli, A.M. Moursi, J.K. Montclare, Modified Tat peptide with cationic lipids enhances gene transfection efficiency via temperature-dependent and caveolae-mediated endocytosis, J. Control. Release 152 (2011) 278–285.
[24] E. Rytting, J. Nguyen, X. Wang, T. Kissel, Biodegradable polymeric nanocarriers for pulmonary drug delivery, Expert Opin. Drug Deliv. 5 (2008) 629–639.
[25] M.T. Tse, C. Blatchford, H. Oya Alpar, Evaluation of different buffers on plasmid DNA encapsulation into PLGA microparticles, Int. J. Pharm. 370 (2009) 33–40.
[26] A.C. Hunter, S.M. Moghimi, Cationic carriers of genetic material and cell death: a mitochondrial tale, Biochim. Biophys. Acta 1797 (2010) 1203–1209.
[27] Y. Cu, W. Saltzman, Drug delivery: stealth particles give mucus the slip, Nat. Mater. 8 (2009) 11–13.
[28] J.S. Suk, S.K. Lai, Y.Y. Wang, L.M. Ensign, P.L. Zeitlin, M.P. Boyle, J. Hanes, The penetration of fresh undiluted sputum expectorated by cystic fibrosis patients by non-adhesive polymer nanoparticles, Biomaterials 30 (2009) 2591–2597.
[29] J. Nguyen, X. Xie, M. Neu, R. Dumitrascu, R. Reul, J. Sitterberg, U. Bakowsky, R. Schermuly, L. Fink, T. Schmehl, T. Gessler, W. Seeger, T. Kissel, Effects of cell-penetrating peptides and pegylation on transfection efficiency of polyethylenimine in mouse lungs, J. Genet. Med. 10 (2008) 1236–1246.
[30] G. Kibria, H. Hatakeyama, N. Ohga, K. Hida, H. Harashima, Dual-ligand modification of PEGylated liposomes shows better cell selectivity and efficient gene delivery, J. Control. Release 153 (2011) 141–148.
[31] K. Oglecka, P. Lundberg, M. Magzoub, L.E. Göran Eriksson, U. Langel, A. Gräslund, Relevance of the N-terminal NLS-like sequence of the prion protein for membrane perturbation effects, Biochim. Biophys. Acta 1778 (2008) 206–213.
[32] R. Sawant, V. Torchilin, Intracellular transduction using cell-penetrating peptides, Mol. Biosyst. 6 (2010) 628–640.