International Journal of Pharmaceutics 392 (2010) 241–248

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Folate-PEG modified poly(2-(2-aminoethoxy)ethoxy)phosphazene/DNA nanoparticles for gene delivery: Synthesis, preparation and in vitro transfection efficiency

Pengcheng Zhang, Zhiwen Zhang, Yongxin Yang, Yaping Li *

Center for Drug Delivery System, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 22 December 2009
Received in revised form 30 January 2010
Accepted 10 March 2010
Available online 16 March 2010

Keywords:
DNA
Poly(phosphazene)
Folic acid
Nanoparticles
Gene

ABSTRACT

Target-specific technique can significantly enhance the efficacy of gene delivery system which was limited by many cellular barriers. In this work, a new folate-PEG modified poly(2-(2-aminoethoxy)ethoxy)phosphazene (PAEP), namely, folate-PEG-PAEP was synthesized as a folate receptor (FR) targeted carrier, and the cytotoxicity, transfection efficiency, cellular uptake and intracellular trafficking of folate-PEG-PAEP/DNA nanoparticles (FPPN) were investigated. Compared with the PAEP/DNA nanoparticles (PN), the cytotoxicity of FPPN decreased significantly at high dose. FPPN showed much higher transfection efficiency (15.85 ± 1.23%) compared with PN (6.71 ± 0.42%) in FR overexpressing Hela cells, but no significant difference was observed in CHO-k1 cells lacking FR. The transfection activity of FPPN could be reversed in the presence of 1.0 mM free folic acid in Hela cells. The cellular uptake of FPPN was 37.38% higher than that of PN in Hela cells. These results indicated that FPPN could be a potential targeted gene delivery system.

1. Introduction

Gene therapy can offer a promising way for the treatment of many diseases such as cancer (El-Aneed, 2004), HIV (Weber et al., 2008), neurodegeneration diseases (Bergen et al., 2008), etc. However, a therapeutic gene has to overcome many physiological barriers to take effect, as a result, a safe and efficient gene delivery system is necessary for successful gene therapy (Rolland, 2005). Recently, nonviral vectors have attracted much attention because of their low safety risk, easy preparation in large quantity and low cost. Unfortunately, most of nonviral vectors suffer from lower gene transfection efficiency compared with viral vectors (Luten et al., 2003; Park et al., 2006; Kawakami et al., 2008).

Poly(phosphazene) could be a candidate vector for efficient gene delivery because of its biodegradability and lower toxicity compared with poly(ethylenimine) (PEI) (Laurencin et al., 1987; Boussif et al., 1995), poly-L-lysine (PLL) (Asayama et al., 1997) and poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) (Van de watering et al., 1997). So far, the great efforts have been made to improve its transfection efficiency by substituting with different side groups, changing molecular weight or conjugating poly(ethylene glycol) and ligand (De Wolf et al., 2005, 2007; Luten et al., 2008; Yang et al., 2008a,b). Although poly(phosphazene) has shown potential advantage for DNA delivery (De Wolf et al., 2005; Yang et al., 2008b), the intracellular trafficking of poly(phosphazene)/DNA nanoparticles has not been explored until today.

In this work, a new poly(phosphazene) derivative, folate-PEG modified poly(2-(2-aminoethoxy)ethoxy)phosphazene (PAEP), namely, folate-PEG-PAEP (FPP) was synthesized. The in vitro transfection efficiency, cytotoxicity, cellular uptake and intracellular trafficking of FPP/DNA nanoparticles (FPPN) were evaluated.

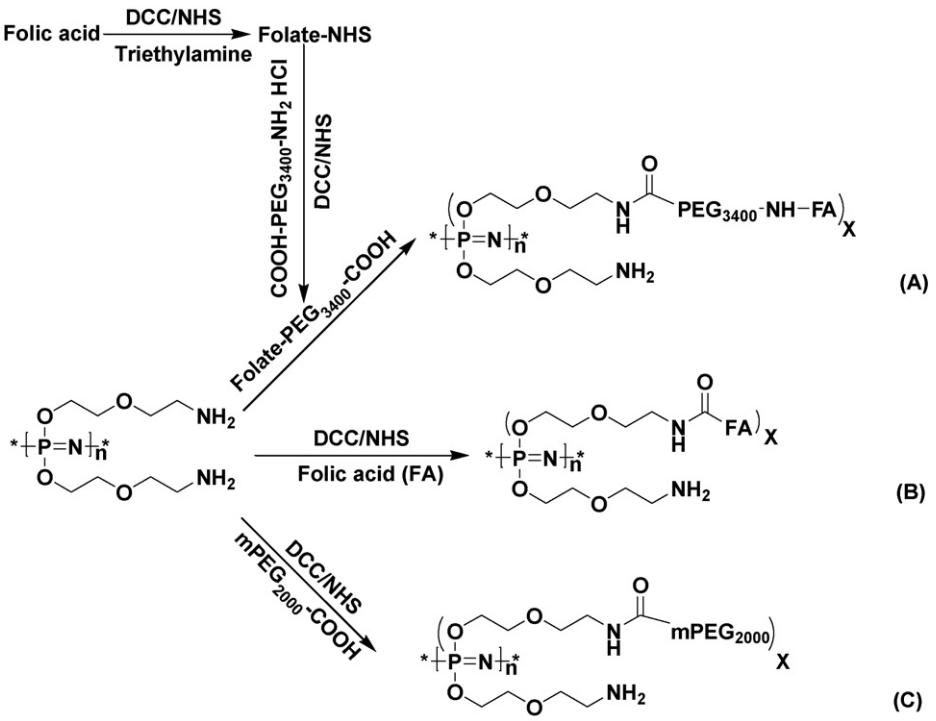
2. Materials and methods

2.1. Materials

All the reagents (analytical grade) were purchased from Shanghai Chemical Reagents Corp., unless otherwise noted. COOH-PEG_{3400}-NH_2·HCl was obtained from Nektar (Huntsville, AL, USA). PEI 25K, poly(ethylene glycol) methyl ether ($mPEG_{2000}$-OH) and folic acid were all purchased from Sigma–Aldrich Co. The Lysotrancker Red Kit, Hoechst 33258 and YOYO-1 were purchased from Molecular Probes (Eugene, OR). Plasmid EGFP-N1 and pGL3 control vector were purchased from Clontech (Palo Alto, CA, USA) and Promega (Madison, WI), respectively. The plasmid DNA (pDNA) was amplified in DH5α strain of *E. coli* and purified by EndFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany).

* Corresponding author. Tel.: +86 21 5080 6820; fax: +86 21 5080 6820.
E-mail address: ypli@mail.shcnc.ac.cn (Y. Li).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.03.030



Scheme 1. Synthesis of FPP (A), FP (B) and PP (C).

2.2. Cell culture

The Hela and CHO-k1 cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were grown in DMEM (Sigma) and RPMI 1640 (Sigma) containing 10% fetal bovine serum (FBS, Gibico), 2 mM L-glutamine, streptomycin (40 μg/ml) and ampicillin (40 U/ml) at 37 °C in a humidified and 5% CO_2 incubator, respectively.

2.3. Synthesis of polymer

The synthesis of PAEP was carried out according to the Allcock's procedure (Allcock and Chang, 1991) and confirmed by a 300 MHz ^{1}H NMR spectrometer (Varian USA) in advance. Three PAEP derivatives were synthesized by coupling folate-PEG_{3400}-COOH, folic acid or $mPEG_{2000}$-COOH to the amino groups of PAEP as shown in Scheme 1.

To synthesize FPP, the carboxyl group of folic acid (110 mg, 0.25 mmol) was activated by N,N′-dicyclohexylcarbodimide (DCC)/N-hydroxysuccinimide (NHS) in 5 ml anhydrous dimethyl sulfoxide (DMSO) at the presence of 0.06 ml triethylamine in dark for 24 h at room temperature. DCC was 1.2-fold over folic acid, and DCC/NHS molar ratio was 1:1. After the dicyclohexylurea (DCU) was filtrated off, the reaction solution containing folate N-hydroxysuccinimidyl ester (folate-NHS) was incubated with COOH-PEG_{3400}-NH_2·HCl (425 mg, 0.125 mmol) for 24 h. The resulting solution was dialyzed against deionized water for 48 h, filtrated and lyophilized to obtain folate-PEG_{3400}-COOH. DCC (19.4 mg, 0.094 mmol), NHS (10.8 mg, 0.094 mmol) and PAEP (400 mg, 3.16 mmol –NH_2) were added to DMSO (20 ml) containing folate-PEG_{3400}-COOH (300 mg, 0.078 mmol), and the mixture was incubated for 24 h at room temperature in dark. The resulting solution was filtrated, and the filtrate was dialyzed against deionized water using regenerated cellulose membranes (MWCO: 7 kDa, Spectrum Co.) for 48 h, and finally lyophilized to obtain FPP. The FPP was confirmed by a 300 MHz ^{1}H NMR spectrometer and a FT-IR spectrometer 750 (Nicolet, USA).

As control, the synthesis of folate-PAEP (FP) was the same as FPP except that folic acid was used to replace folate-PEG_{3400}-COOH. To obtain PEG-PAEP (PP), $mPEG_{2000}$-COOH was synthesized from $mPEG_{2000}$-OH according to the literature (Chen et al., 2003). $mPEG_{2000}$-COOH was coupled to PAEP following the same protocol as the synthesis of FPP. FP and PP were confirmed by a 300 MHz ^{1}H NMR spectrometer. The average molecular weights (Mw) and polydispersity index (PDI = Mw/Mn) of PAEP, FP, PP and FPP were determined by gel permeation chromatography (GPC) on a Waters 2695 GPC system equipped with Ultrahydrogel columns (250 PKGD and 1000 PKGD) and a refractive index detector (model 2414). 0.05% aqueous sodium azide solution as mobile phase with a flow rate of 0.5 ml/min was used. The samples were analyzed and calibrated by dextran standards (Mp = 4400–401,000 Da, American Polymer Standards Corporation, USA).

2.4. Preparation and characteristics of polymer/DNA nanoparticles

The FPP/DNA nanoparticles (FPPN) were prepared by vortexing polymer solution with EGFP-N1 solution for 30 s at various N/P ratios, and incubated for 30 min at room temperature before use. To prepare nanoparticles used in cellular uptake and subcellular colocalization, EGFP-N1 was replaced with pGL3 labeled with YOYO-1 at a ratio of 1 dye molecule to 300 bp. The DNA condensation ability of polymer was investigated via electrophoresis for 45 min at 110 V/cm in a Tris-acetate–EDTA buffer system (pH 8.0) using naked DNA as control, and DNA was visualized by ethidium bromide staining. The size distribution and ζ-potential of nanoparticles at optimal N/P ratio for transfection were measured in water by laser light scattering using a Nicomp 380/ZLS zeta potential analyzer (Particle Sizing System, USA). As control, PEI/DNA nanoparticles (PEN), PAEP/DNA nanoparticles (PN), FP/DNA nanoparticles (FPN), and PP/DNA nanoparticles (PPN) also were prepared according to the same procedure as FPPN.

2.5. Cell viability

The MTT method was applied to evaluate the cytotoxicity of nanoparticles, and PEI 25 K was used as control. Cells were seeded (2×10^4 cells/well) in 96-well microtitre plates, and grew for 24 h before the growth media were replaced with 200 μl fresh DMEM or RPMI 1640 medium containing 10% FBS and different amount of nanoparticles (2.0, 5.0, 10.0 or 20.0 μg/ml DNA) with the N/P ratio 15 for CHO-k1 cells and 25 for Hela cells, respectively. After 3 h incubation, the medium was replaced with 100 μl of growth medium containing 1 mg/ml MTT, and incubated for 4 h further. Thereafter, the medium was carefully removed, and 150 μl of DMSO was added to dissolve the crystals formed by living cells. Absorbance was measured at 570 nm using a microplate reader (Infinite 2000, TECAN). The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability}\,(\%) = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}}$$

$\text{OD}_{\text{sample}}$, $\text{OD}_{\text{control}}$ represented absorption from the cells treated with nanoparticles and PBS, and OD_{blank} represented absorption from the cells not treated with MTT.

2.6. In vitro transfection

The transfection efficiency of nanoparticles was evaluated on Hela cells and CHO-k1 cells using EGFP-N1 as the model DNA. These two cell lines were chosen, because the FR expression levels of them were different. Hela cells were characterized as FR overexpressing cells (Leamon and Low, 1993; Rosenholm et al., 2009), but CHO-k1 cells were devoid of FR (Paranjpe et al., 2004; Stevens et al., 2004). The cells were seeded in 24-well plates at 1×10^5 cells/well and grown for 24 h to achieve 60–70% confluence. The cells were accustomed for 30 min in 450 μl transfection media (DMED or RPMI 1640 with 10% FBS without antibiotics, 1.0 mM folic acid was added in half of the wells for the competition experiment). Thereafter, the cells were transfected for 3 h by adding 50 μl nanoparticles containing 2.5 μg DNA with the optimal N/P ratios 15 and 25 for CHO-k1 and Hela cells, respectively, and incubated for another 45 h in fresh complete medium. Naked DNA was used as control, and all transfection experiments were conducted in triplicate. The enhanced green fluorescent protein (EGFP) expressing cells were visualized using fluorescence inversion microscope system (Olympus IX71) and quantified by a fluorescence activated cell sorter (FACSCalibur, Becton Dickinson, USA).

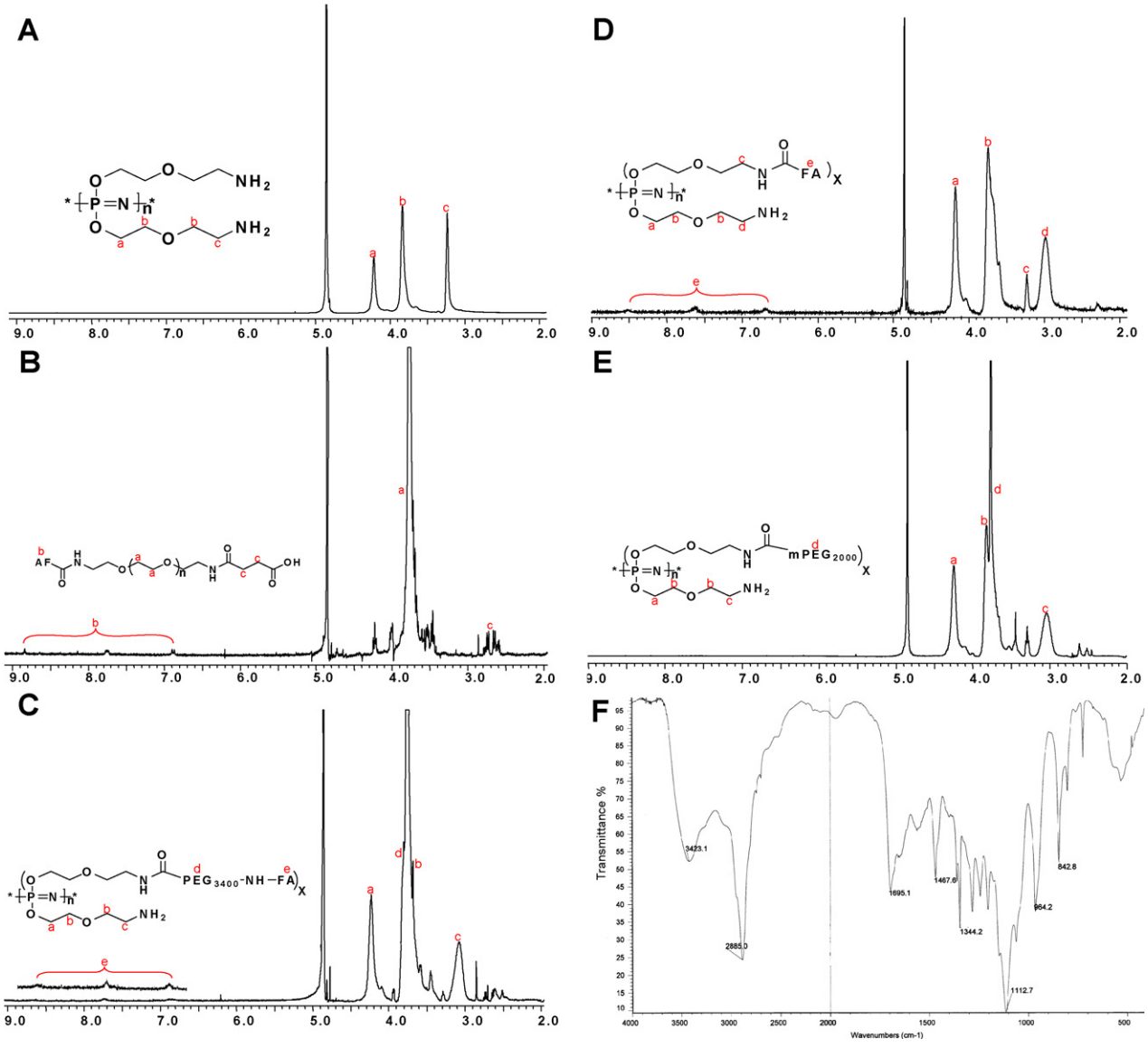


Fig. 1. ^{1}H NMR spectrums of PAEP (A), folate-PEG_{3400}-COOH (B), FPP (C), FP (D), PP (E) in D_2O, and FT-IR spectrum of FPP (F).

Table 1
Molecular weight and polydispersity index of polymer.

Polymers	Mw (kDa)	PDI
PAEP	13.9	1.36
FP	14.5	1.42
PP	19.6	1.87
FPP	24.9	1.96

2.7. Cellular uptake experiment

Hela cells were seeded in 24-well plates (1×10^5 cells/well), and further incubated for 24 h. The cells were accustomed for 30 min in 450 μl transfection medium and transfected for 3 h by adding 50 μl nanoparticles. Then, cells were incubated with 200 μl trypan blue (0.4% in PBS 7.4) for 2 min to quench the extra-cellular fluorescence, washed twice with PBS, collected and finally resuspended in PBS (pH 7.4). The fluorescence was measured using FACSCalibur system. All experiments were performed in triplicate.

2.8. Sub-cellular localization of pDNA

For the sub-cellular localization experiment, cells were seeded on 10 mm^2 glass coverslips coated with a cell-adhesion polymer (PLL) in 24-well plates (1×10^5 cells/well), and incubated for 24 h. The cells were accustomed for 30 min in 450 μl transfection medium, transfected for 3 h by adding 50 μl nanoparticles, and stained for 10 min in 500 μl complete medium containing 10 μg/ml Hoechst 33258 and 2 μM Lysotracker Red. The cells were then incubated with 200 μl trypan blue (0.4% in PBS 7.4) for 2 min to quench the extra-cellular fluorescence, washed twice with PBS, and fixed with 4% paraformaldehyde for 20 min in dark. The cells were mounted on glass slides with 3 μl of MobiGlow (MoBiTec, Goettingen, Germany) and visualized by confocal microscopy (Leica Microsystems).

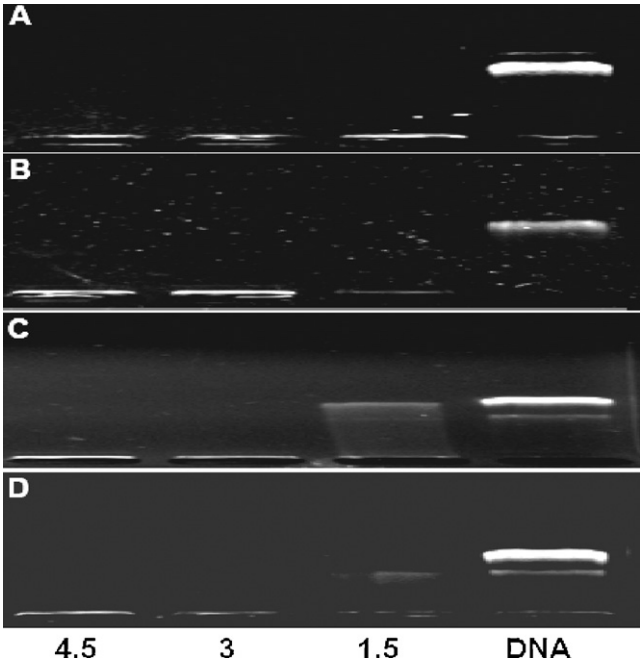


Fig. 2. The DNA retardation assay of PN (A), FPN (B), PPN (C) and FPPN (D) by agarose gel electrophoresis using 1% agarose in Tris-acetate running buffer (pH 8.0). 1.5, 3 and 4.5 indicated the N/P ratios of complexes.

Table 2
Particle size and ζ-potential of nanoparticles with N/P ratio 15 and 25 in distilled water ($n = 3$).

	Particle size (nm)		ζ-Potential (mV)	
	15	25	15	25
PN	96.4 ± 5.5	113.2 ± 7.7	13.73 ± 1.32	18.12 ± 1.70
FPN	110.8 ± 7.6	130.9 ± 8.7	11.19 ± 1.12	16.20 ± 1.14
PPN	97.8 ± 4.3	125.5 ± 8.9	8.62 ± 0.77*	11.90 ± 0.93*
FPPN	88.5 ± 3.9	110.3 ± 7.1	7.76 ± 0.51*	7.69 ± 0.73*

* $p < 0.01$ compared with PN.

2.9. Statistical analysis

Statistical analysis was performed using Student's *t*-test. The differences were considered significant for $p < 0.05$, and very significant for $p < 0.01$.

3. Results and discussion

3.1. Synthesis of polymer

Confirmed by ^{1}H NMR spectrometer, PAEP and folate-PEG_{3400}-COOH were synthesized successfully (Fig. 1A and B). To investigate

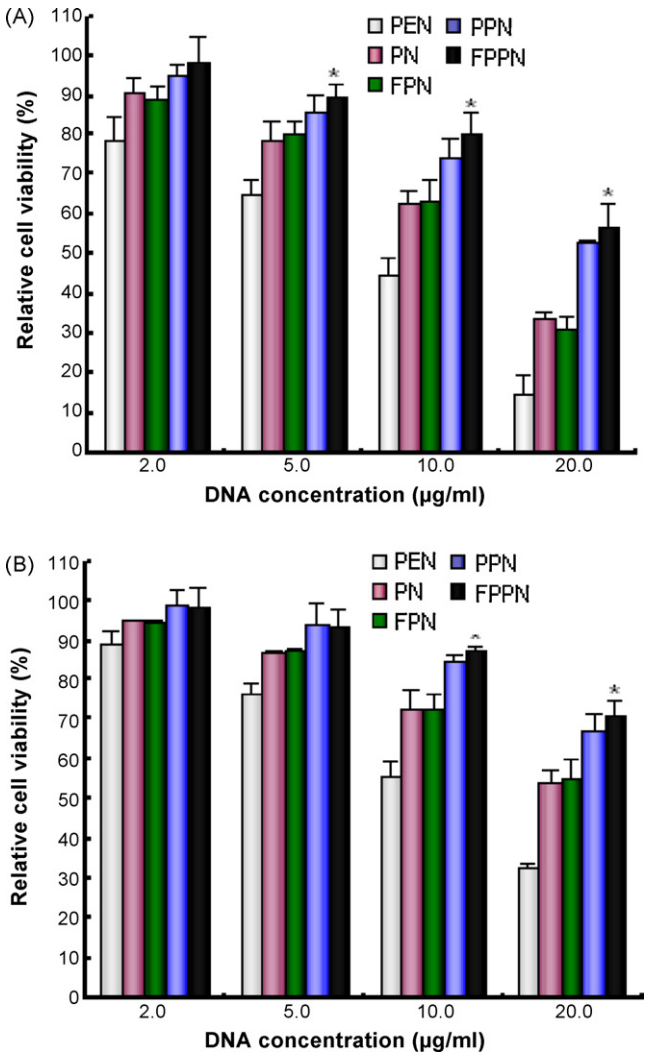


Fig. 3. Relative cell viability of PEC, PN, FPN, PPN or FPPN in Hela cells (A) and CHO-k1 cells (B). The N/P ratio was 25 for Hela cells and 15 for CHO-k1 cells. *$p < 0.05$ compared with PN.

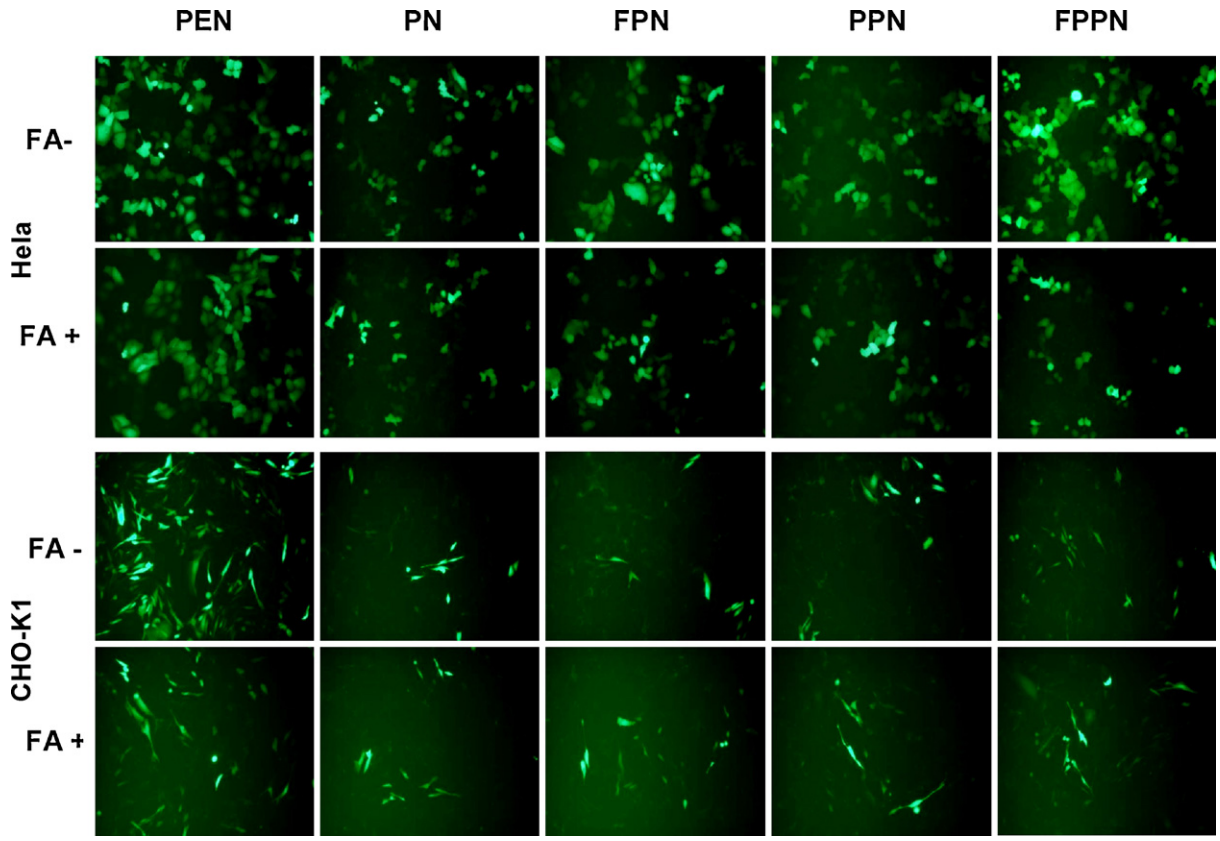


Fig. 4. Fluorescent images of Hela and CHO-k1 cells transfected with PEN, PN, FPN, PPN and FPPN in the absence or presence of 1.0 mM free folic acid.

the effect of PEG and folic acid modification on the transfection efficiency of PAEP, three PAEP derivatives were synthesized by coupling folate-PEG_{3400}-COOH, folic acid or $mPEG_{2000}$-COOH to the amino groups of PAEP. The substitution degree of the PAEP derivatives was controlled by the molar feed ratio and calculated via the integration ratio of peaks at 2.6–2.7 ($-COCH_2CH_2CO-$) with those at 2.8–3.1 ($-CH_2NH_2-$) for FPP in Fig. 1C, peaks at 3.2–3.3 ($-CH_2NHCO-$) with those at 2.8–3.1 ($-CH_2NH_2-$) for FP in Fig. 1D, and peaks at 2.6–2.7 ($-COCH_2CH_2CO-$) with those at 2.8–3.1 ($-CH_2NH_2-$) for PP in Fig. 1E. The substitution degrees of primary amines of PAEP were from 1.43% to 1.56% for FPP, from 1.73% to 1.95% for FP and from 1.56% to 1.71% for PP, respectively. The authenticity of FPP was further confirmed by the infrared spectrum. The absorbance at 2885.0 cm^{-1} was the characteristic absorbance of CH_2 due to the presence of PAEP and PEG. The P=N absorbance at 1250.0 cm^{-1} demonstrated the presence of PAEP. The absorbance at 1695.1 and 842.8 cm^{-1} from C=O and phenyl group, respectively, demonstrated the presence of FA-PEG_{3400} (Fig. 1F). FPP showed good solubility in water (>100 mg/ml) with the hydrolysis half life (at about 270 h). The Mw and PDI of polymer PAEP determined by GPC were 13.9 kDa and 1.36, respectively. PEGylation increased the Mw and PDI of polymer as described in Table 1.

3.2. Physicochemical characteristics of nanoparticles

DNA condensation ability of cationic polymer plays a crucial role in protecting DNA from degradation. To investigate whether the conjugation affects the DNA condensation ability of PAEP, a series of nanoparticles with different N/P ratios was prepared and evaluated by gel retardation assay. As shown in Fig. 2, the DNA condensation ability of FP and PAEP did not show significant difference ($p > 0.05$, Fig. 2A and B), but the PEGylation of PAEP slightly decreased the DNA condensation ability of the polymer (Fig. 2C and D). Although the PEGylation interfered with the compactness of polymer/DNA nanoparticles as previous report (Erbacher et al., 1999), the N/P ratio applied in our experiments was over 3, at which FPP or PP could condense plasmid DNA very well.

Nanoparticles at N/P ratio 15 and 25 were prepared for the following experiments, because the transfection activity of nanoparticles at this N/P on CHO-k1 and Hela cells was the highest according to our preliminary experiment. The mean particle sizes and ζ-potentials of nanoparticles were shown in Table 2. The particle sizes of four nanoparticles did not demonstrate significant difference, but the ζ-potentials of PPN and FPPN were sharply decreased compared with those of PN and FPN ($p < 0.01$), which indicated that PEGylation of the nanoparticles could shield the surface charge. In addition, PEGylation could also significantly reduce the opsonization phagocyte recognition, and prolong particle circulation time through surface charge mask and steric shield effect (Zahr et al., 2006), which favor further in vivo application such as increasing the distribution of nanoparticles in tumor (Dobrovolskaia et al., 2008).

3.3. Cell viability

The cytotoxicity is one of the obstructions for successful gene delivery, thus the cytotoxicity of polymer/DNA complexes was evaluated by MTT method. Compared with PEN, PN showed lower toxicity when nanoparticles containing 1.0–4.0 μg DNA were added to Hela and CHO-k1 cells ($p < 0.01$) (Fig. 3). As shown in Fig. 3, the cytotoxicity of FPPN was significantly lower compared with that of PN at same dose, when nanoparticles containing 1.0–4.0 μg DNA were added to Hela cells ($p < 0.05$), which showed that the PEGylation of the PAEP could decrease the cytotoxicity further. The same phenomenon was observed when nanoparticles containing 2.0–4.0 μg DNA were added to CHO-k1 cells ($p < 0.05$). As the cyto-

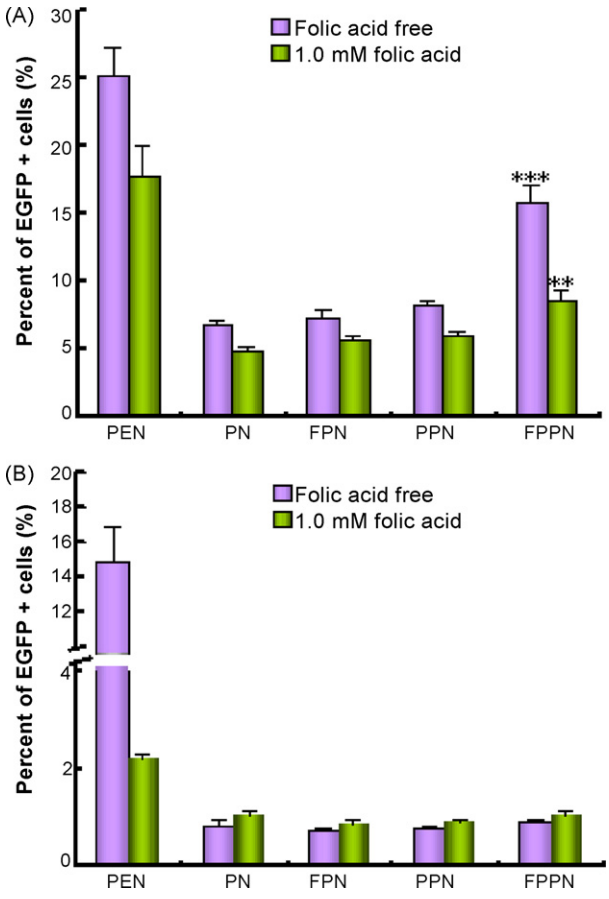


Fig. 5. Transfection efficiency of nanoparticles on Hela cells (A) and CHO-k1 cells (B) in the absence and presence of free folic acid. The N/P ratio was 25 for Hela cells and 15 for CHO-k1 cells, and PEC at the same N/P ratio was used as control. $^{**}p < 0.01$ compared with FPPN in folic acid free transfection medium, $^{***}p < 0.001$ compared with PN.

toxicity of cationic polymers mainly resulted from the electrostatic interaction between nanoparticles and cell membranes (Kunath et al., 2003; Qiu and Bae, 2007), the low ζ-potentials of FPPN could contribute to their low cytotoxicity.

3.4. In vitro transfection

Receptor targeted gene delivery is an effective way to overcome the plasma membrane of specific cells (Russ and Wagner, 2007), and folic acid is one of the ideal ligands for targeted gene delivery (Lu and Low, 2002). The transfection efficiency of PN, FPN, PPN and FPPN were investigated on Hela and CHO-k1 cells. Transfected cells were visualized using fluorescent microscope (Fig. 4) and quantified by FACS (Fig. 5). The transfection efficiencies of PN, FPN and PPN in Hela cells did not show significant difference ($p > 0.05$), but FPPN showed approximately one time higher transfection activity compared with other nanoparticles ($p < 0.001$), which indicated that the modification of folate-PEG-COOH could significantly increase the transfection efficiency. In the presence of excess free folic acid (1.0 mM), the transfection efficiency of FPPN decreased from $15.85 \pm 1.23\%$ to $8.43 \pm 0.92\%$ (Fig. 5A). Interestingly, the transfection efficiencies of non-FR target PN and PPN decreased slightly by adding free folic acid, and the precise mechanism is still under investigation in our lab. As the FR located lipid rafts are abundant of some specific lipids (Kamen and Smith, 2004), one possible mechanism could be as follow: the presence of free folic acid induced the endocytosis of FR, and further changed the lipid composition of cell membrane, finally changed the ionic

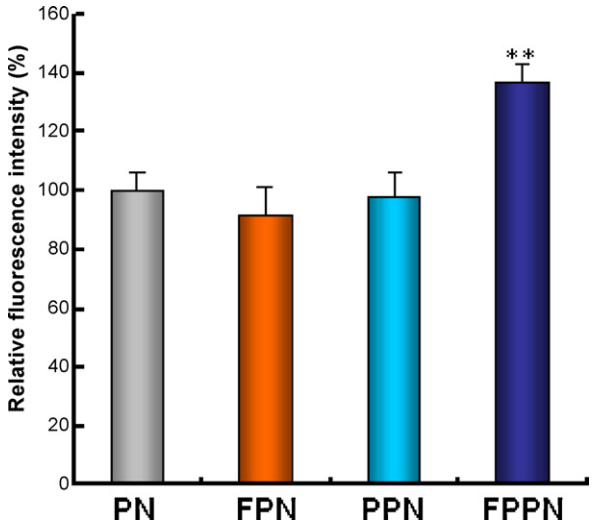


Fig. 6. Cellular uptake of pDNA by Hela cells in the form of PN, FPN, PPN or FPPN. $^{*}p < 0.05$ compared with PN.

interaction between complexes and cell membrane, which could also be an optional explanation for the different responses to non-FR targeted nanoparticles between Hela cells and CHO-k1 cells at the presence of free folic acid. In CHO-k1 cells, FPPN was not superior to other nanoparticles in the presence or absence of free folic acid, which indicated that the presence of FR on cell membrane was a very important factor for high transfection efficiency of FPPN.

3.5. Cellular uptake

In order to explore the effect of FR-dependent endocytosis pathway on cellular uptake of plasmid DNA, the amounts of DNA endocytosed by Hela cells were quantified by FACS. According to Fig. 6, the cellular uptake of FPPN rose by 37.38% compared with PN ($p < 0.01$), on the contrary, FPN was not superior to PN on cellular uptake. The difference could result from the difference of their physical properties, because the ζ-potential of FPPN was significantly lower than that of FPN and PN (Table 1). It has been reported that the higher ζ-potential would result in more non-specific interaction between nanoparticles and serum or cell membrane (Zahr et al., 2006), which could consequently bring about the shielding of folic acid by serum proteins and non-specific endocytosis involving ionic interaction. Thus, nearly no FPN was endocytosed through FR-dependent pathway. After PEGylation, the cellular uptake of PPC did not decrease with ζ-potential decreasing (Fig. 6), which could be due to the low PEG substitution degree (Mok et al., 2009). These results indicated that the FR-dependent endocytosis pathway played an important role in cellular uptake and transfection efficiency of FPPN.

3.6. Sub-cellular colocalization

Since FACS cannot discriminate the localization of nanoparticles in cells, confocal microscopy was applied to investigate the colocalization of nanoparticles with lysosomes (Fig. 7). The uptake of plasmid DNA into Hela cells was observed by the presence of green dots from YOYO-1 labeled pDNA in the cytoplasm around the nucleus (blue structures from Hoechst staining). As the degradation of DNA in lysosome is one of the obstructions for efficient gene delivery, we focused on the colocalization of lysosomes (red dots from Lysotracker Red staining) with endocytosed plasmid DNA. As shown in Fig. 7, FPN (Fig. 7B) or PPN (Fig. 7C) showed

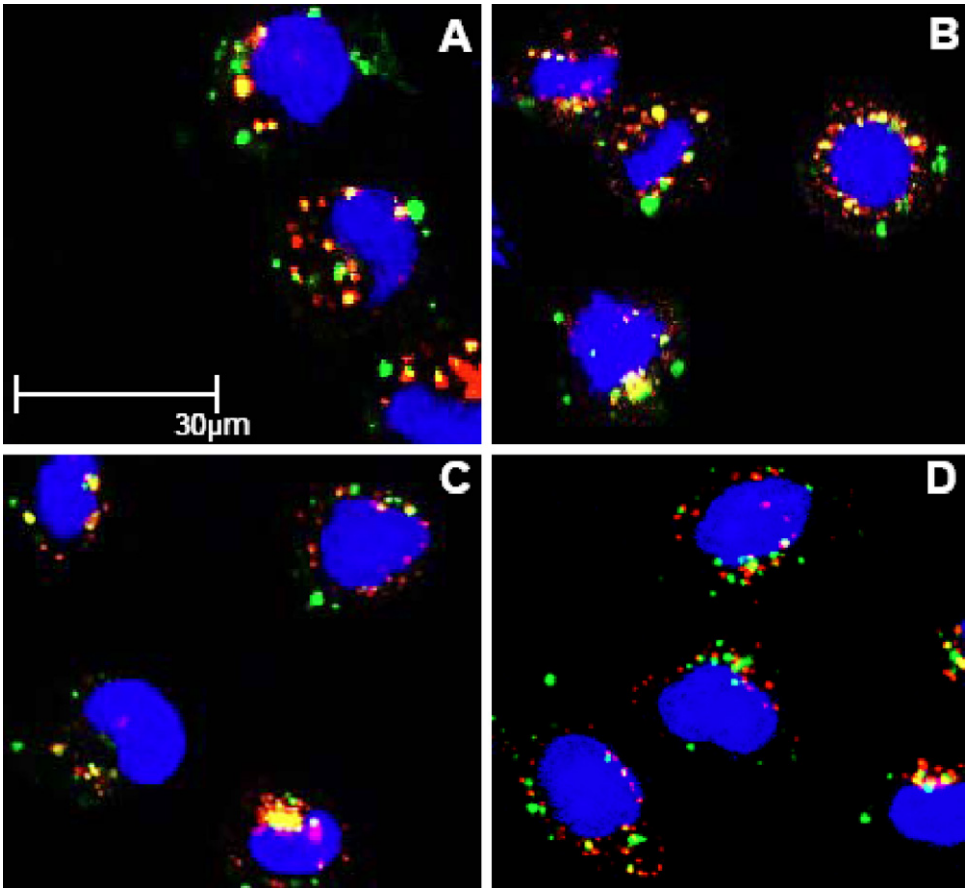


Fig. 7. Sub-cellular localization of uptaked pDNA in Hela cells treated with PN (A), FPN (B), PPN (C) or FPPN (D) visualized by confocal microscopy. Nuclei (blue), lysosomes (red) and pDNA (green) were stained with Hoechst, lysotracker Red, and YOYO-1, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

no obvious difference with PN (Fig. 7A) in the localization of plasmid DNA and most of endocytosed plasmid DNA was co-localized with lysosomes. On the contrary, the sub-cellular localization of FPPN slightly changed, less pDNA was co-localized with lysosomes (Fig. 7D). For folate modified drug carriers, it is generally assumed that a triggering mechanism must occur to release the drug from endosomes (Rui et al., 1998; Kim et al., 2008). The FPPN, lacking purposely designed triggering mechanism, seemed to show lysosomal escape ability to some extent (Fig. 7), which was also observed from microgels by Lyon's group (Nayak et al., 2004). Lysosome escape of pDNA due to endosomal sorting process could be one of the reasons for enhanced transfection efficiency (Maxfield and McGraw, 2004), which supplemented the effect of increased cellular uptake of pDNA through FR-dependent endocytosis in enhanced tranfection efficiency.

4. Conclusion

The folate-PEG-PAEP was synthesized as a folate receptor targeted carrier. The cytotoxicity of FPPN decreased significantly at high dose compared with the PAEP/DNA nanoparticles. FPPN showed much higher transfection efficiency in FR overexpressing Hela cells, but no significant difference was observed in CHO-k1 cells lacking FR. The transfection activity of FPPN could be reversed in the presence of 1.0 mM free folic acid in Hela cells. The cellular uptake of FPPN was 37.38% higher than that of PN in Hela cells. These results suggested that folate-PEG-PAEP could be a promising gene delivery system.

Acknowledgments

The National Basic Research Program of China (2010CB934000 and 2007CB935800), the National Natural Science Foundation of China (30925041), National Science & Technology Major Project "Key New Drug Creation and Manufacturing Program" (Nos. 2009ZX09501-024 and 2009ZX09301-001), and Shanghai Nanomedicine Program (0852nm05700) are gratefully acknowledged for financial support.

References

Allcock, H.R., Chang, J.Y., 1991. Poly(organophosphazenes) with oligopeptides as side groups-prospective biomaterials. Macromolecules 24, 993–999.
Asayama, S., Maruyama, A., Cho, C.S., Akaike, T., 1997. Design of comb-type polyamine copolymers for a novel pH-sensitive DNA carrier. Bioconjugate Chem. 8, 833–838.
Bergen, J.M., Park, I.K., Horner, P.J., Pun, S.H., 2008. Nonviral approaches for neuronal delivery of nucleic acids. Pharm. Res. 25, 983–998.
Boussif, O., Lezoualch, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonuceotide transfer into cells in culture and in-vivo: to polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7301–9297.
Chen, W.N., Luo, W.J., Wang, S.G., Bei, J.Z., 2003. Synthesis and properties of poly(L-lactide)-poly (ethylene glycol) multiblock copolymers by coupling triblock copolymers. Polym. Adv. Technol. 14, 245–253.
De Wolf, H.K., Luten, J., Snel, C.J., Oussoren, C., Hennink, W.E., Storm, G., 2005. In vivo tumor transfection mediated by polyplexes based on biodegradable poly(DMAEA)-phosphazene. J. Control. Release 109, 275–287.
De Wolf, H.K., de Raad, M., Snel, C., van Steenbergen, M.J., Fens, M.H.A.M., Storm, G., Hennink, W.E., 2007. Biodegradable poly(2-dimethylamino ethylamino)phosphazene for in vivo gene delivery to tumor cells. Effect of polymer molecular weight. Pharm. Res. 24, 1572–1580.

Dobrovolskaia, M.A., Aggarwal, P., Hall, J.B., McNeil, S.E., 2008. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution. Mol. Pharm. 5, 487–495.
El-Aneed, A., 2004. An overview of current delivery systems in cancer gene therapy. J. Control. Release 94, 1–14.
Erbacher, P., Bettinger, T., Belguise-Valladier, P., Zou, S.M., Coll, J.L., Behr, J.P., Remy, J.S., 1999. Transfection and physical properties of various saccharide, poly(ethylene glycol), and antibody-derivatized polyethylenimines (PEI). J. Gene Med. 1, 210–222.
Kawakami, S., Higuchi, Y., Hashida, M., 2008. Nonviral approaches for targeted delivery of plasmid DNA and oligonucleotide. J. Pharm. Sci. 97, 726–745.
Kamen, B.A., Smith, A.K., 2004. A review of folate receptor alpha cycling and 5-methyltetrahydrofolate accumulation with an emphasis on cell models in vitro. Adv. Drug Deliv. Rev. 56, 1085–1097.
Kim, D., Lee, E.S., Park, K., Kwon, I.C., Bae, Y.H., 2008. Doxorubicin loaded pH-sensitive micelle: antitumoral efficacy against ovarian A2780/DOXR tumor. Pharm. Res. 25, 2074–2082.
Kunath, K., von Harpe, A., Fischer, D., Peterson, H., Bickel, U., Voigt, K., Kissel, T., 2003. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. J. Control. Release 89, 113–125.
Laurencin, C.T., Koh, H.J., Neenan, T.X., Allcock, H.R., Langer, R., 1987. Controlled release using a new bioerodible polyphosphazene matrix system. J. Biomed. Mater. Res. Part A 21, 1231–1246.
Leamon, C.P., Low, P.S., 1993. Membrane folate-binding proteins are responsible for folate protein conjugate endocytosis into cultured-cells. Biochem. J. 291, 855–860.
Lu, Y.J., Low, P.S., 2002. Folate-mediated delivery of macromolecular anticancer therapeutic agents. Adv. Drug Deliv. Rev. 54, 675–693.
Luten, J., van Steenis, J.H., van Someren, R., Kemmink, J., Schuurmans-Nieuwenbroek, N.M.E., Koning, G.A., Crommelin, D.J.A., van Nostrum, C.F., Hennink, W.E., 2003. Water-soluble biodegradable cationic polyphosphazenes for gene delivery. J. Control. Release 89, 483–497.
Luten, J., van Steenbergen, M.J., Lok, M.C., de Graaff, A.M., van Nostrum, C.F., Talsma, H., Hennink, W.E., 2008. Degradable PEG-folate coated poly(DMAEA-co-BA)phosphazene-based polyplexes exhibit receptor-specific gene expression. Eur. J. Pharm. Sci. 33, 241–251.
Maxfield, F.R., McGraw, T.E., 2004. Endocytic recycling. Nat. Rev. Mol. Cell Biol. 5, 121–132.
Mok, H., Bae, K.H., Ahn, C.H., Park, T.G., 2009. PEGylated and MMP-2 specifically DeP-EGylated quantum dots: comparative evaluation of cellular uptake. Langmuir 25, 1645–1650.
Nayak, S., Lee, H., Chmielewski, J., Lyon, L.A., 2004. Folate-mediated cell targeting and cytotoxicity using thermoresponsive microgels. J. Am. Chem. Soc. 126, 10258–10259.
Paranjpe, P.V., Chen, Y., Kholodovych, V., Welsh, W., Stein, S., Sinka, P.J., 2004. Tumor-targeted bioconjugate based delivery of camptothecin: design, synthesis and in vitro evaluation. J. Control. Release 100, 275–292.
Park, T.G., Jeong, J.H., Kim, S.W., 2006. Current status of polymeric gene delivery systems. Adv. Drug Deliv. Rev. 58, 467–486.
Qiu, L.Y., Bae, Y.H., 2007. Self-assembled polyethylenimine-graft-poly(epsilon-caprolactone) micelles as potential dual carriers of genes and anticancer drugs. Biomaterials 28, 4132–4142.
Rolland, A., 2005. Gene medicines: the end of the beginning? Adv. Drug Deliv. Rev. 57, 669–673.
Rosenholm, J.M., Meinander, A., Peulhu, E., Niemi, R., Eriksson, J.E., Sahlgren, C., Linden, M., 2009. Targeting of porous hybrid silica nanoparticles to cancer cells. ACS Nano 3, 197–206.
Rui, Y.J., Wang, S., Low, P.S., Thompson, D.H., 1998. Diplasmenylcholine-folate liposomes: an efficient vehicle for intracellular drug delivery. J. Am. Chem. Soc. 120, 11213–11218.
Russ, V., Wagner, E., 2007. Cell and tissue targeting of nucleic acids for cancer gene therapy. Pharm. Res. 24, 1047–1057.
Stevens, P.J., Sekido, M., Lee, R.J., 2004. A folate receptor-targeted lipid nanoparticle formulation for a lipophilic paclitaxel prodrug. Pharm. Res. 21, 2153–2157.
Van de watering, P., Cherng, J.Y., Talsma, H., Hennink, W.E., 1997. Relation between transfection efficiency and cytotoxicity of poly(2-(dimethylamino)ethyl methacrylate)/plasmid nanoparticles. J. Control. Release 49, 59–69.
Weber, N., Ortega, P., Clemente, M.I., Shcharbin, D., Bryszewska, M., de la Mata, F.J., Gomez, R., Munoz-Fernandez, M.A., 2008. Characterization of carbosilane dendrimers as effective carriers of siRNA to HIV-infected lymphocytes. J. Control. Release 132, 55–64.
Yang, Y.X., Xu, Z.H., Jiang, J.G., Gao, Y., Gu, W.W., Chen, L.L., Tang, X.Z., Li, Y.P., 2008a. Poly(imidazole/DMAEA)phosphazene/DNA self-assembled nanoparticles for gene delivery: synthesis and in vitro transfection. J. Control. Release 127, 273–279.
Yang, Y.X., Xu, Z.H., Chen, S.W., Gao, Y., Gu, W.W., Chen, L.L., Pei, Y.Y., Li, Y.P., 2008b. Histidylated cationic polyorganophosphazene/DNA self-assembled nanoparticles for gene delivery. Int. J. Pharm. 353, 277–282.
Zahr, A.S., Davis, C.A., Pishko, M.V., 2006. Macrophage uptake of core–shell nanoparticles surface modified with poly(ethylene glycol). Langmuir 22, 8178–8185.